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เอกสารแนบหมายเลข 1

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ORIGINAL PAPER

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

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Abstract Electrophoretic protein profiles of female salivary glands of five sibling species within the *Anopheles barbirostris* complex, namely *A. barbirostris* species A1 (Forms A, B, and D), A2, A3, and A4 and *Anopheles campestris*-like (Forms B and E), were analyzed. At least eight major and several minor protein bands were detected in the glands of each species, of which each morphological region contained different major proteins. The protein profiles distinguished the five sibling species. The variability in major proteins among species was observed in the 40–48, 32–37, and 10–18 kDa ranges. No difference in protein profiles was found in different cytogenetic forms. Polymorphism of the protein profiles within species was only noted in species A4. The lowest major protein (marker) band of each species showed remarkably different relative mobility on SDS–polyacrylamide gels. NanoLC-MS analysis revealed that the marker protein of some species matched with a protein involving in blood feeding, gSG6, of *Anopheles gambiae* and *Anopheles freeborni*. 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.

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Introduction

Malaria remains a major world health problem, particularly in the tropics (WHO 1999). Anopheline mosquitoes are the exclusive vectors of human malaria. Salivary glands are of interest in anopheline mosquitoes because transmission of malaria to vertebrate hosts depends on the ability of *Plasmodium* sporozoites to invade the salivary glands of female vector mosquitoes. The female salivary glands secrete a wide array of compounds that are delivered with the saliva and help blood feeding by affecting the host's hemostatic response. Carbohydrate hydrolyzing enzymes in saliva also helps the digestion of sugar. Additionally, the saliva is a vehicle for pathogens and may also enhance or facilitate infectivity during blood meals (Ribeiro and Francischetti 2003). Analyses of salivary gland proteins (proteomes) and/or genes (transcriptomes) were performed in a number of *Anopheles* mosquitoes (Arca et al. 1999a, b, 2005; Calvo et al. 2004, 2007, 2009; Francischetti et al. 2002; Lanfrancotti et al. 2002; Valenzuela et al. 2003). These studies led to the discovery of several

novel protein families, providing some clues to the evolution of blood feeding and revealing the complexity of mosquito salivary secretions. Calvo et al. (2004, 2009) suggested that genes encoding secreted products in salivary gland of *Anopheles darlingi* (subgenus *Nyssorhynchus*) and *Anopheles gambiae* (subgenus *Cellia*) are evolving rapidly in comparison with housekeeping genes. Valenzuela et al. (2003) found similar results when the salivary gland transcriptomes of *Anopheles stephensi* (subgenus *Cellia*) and *A. gambiae* were compared. These results support the hypothesis that secreted gene products may be good markers for assessing phylogeny among closely related species, as has been demonstrated with triatomine bugs using salivary heme proteins (Soares et al. 1998, 2000).

In Thailand, at least four formally described species of almost identical morphology in the *Barbirostris* subgroup of the subgenus *Anopheles* are recorded, namely *Anopheles barbirostris* Van der Wulp, *Anopheles campestris* Reid, *Anopheles donaldi* Reid, and *Anopheles hodgkini* Reid (Reid 1962, 1968; Harrison and Scanlon 1975; Harbach 2004; Rattanarithikul et al. 2006). Among these species, *A. barbirostris* and/or *A. campestris* were incriminated as a potentially natural vector of *Plasmodium vivax* in Aranyaprathet district, Sa Kaeo Province, southeastern Thailand (Limrat et al. 2001; Apiwathnasorn et al. 2002). Additionally, they were considered as important vectors for increasing malaria cases of *P. vivax* in Thailand (Sattabongkot et al. 2004). Recently, cytogenetics study and molecular analysis of different populations of *A. barbirostris* s.l. in Thailand revealed two distinct species, i.e., *A. barbirostris* (Forms A: X_1, Y_1; B: X_1, X_2, Y_2; and C: X_2, Y_3) and *A. campestris*-like species (Forms B: X_2, Y_2 and E: X_2 Y_5; Saeung et al. 2007). Subsequently, four sibling species with identical mitotic karyotypes of Form A (X_1, X_2, and Y_1) were discovered in the taxon *A. barbirostris*, namely species A1 (Chiang Mai), species A2 (Phetchaburi), species A3 (Kanchanaburi; Saeung et al. 2008), and species A4 (Chiang Mai; Suwannamit et al. 2009). In this study, therefore, electrophoretic protein profiles of female salivary glands of each form and sibling species in the *A. barbirostris* complex were analyzed. Our initial findings revealed differences in the electrophoretical protein profiles. The marker protein bands of the female salivary glands in the five sibling species were also analyzed by nano-liquid chromatography–mass spectrometry (NanoLC-MS).

Materials and methods

Mosquito collection and isoline colonization

Wild-caught, fully engorged females of the *A. barbirostris* complex were collected during June 2008 to June 2009 in eight provinces in Thailand (Fig. 1 and Table 1), where different populations of the *A. barbirostris* complex were reported (Saeung et al. 2007, 2008; Suwannamit et al. 2009). Collection of wild-caught females was performed using both human-baited and animal-baited traps. The live females were transported to the laboratory of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, for isoline colonization using the techniques described by Choochote et al. (1983) and Kim et al. (2003).

Mitotic karyotype

Metaphase chromosomes were prepared from the early fourth-instar larval brains of F_1 and/or F_2 progenies of each isoline using the method of Baimai et al. (1995) and Saeung et al. (2007, 2008).

Identification of mosquito species using rDNA ITS2 region as a molecular marker

Genomic DNA was extracted from a pool of five larvae from each isoline using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. The rDNA ITS2 region was

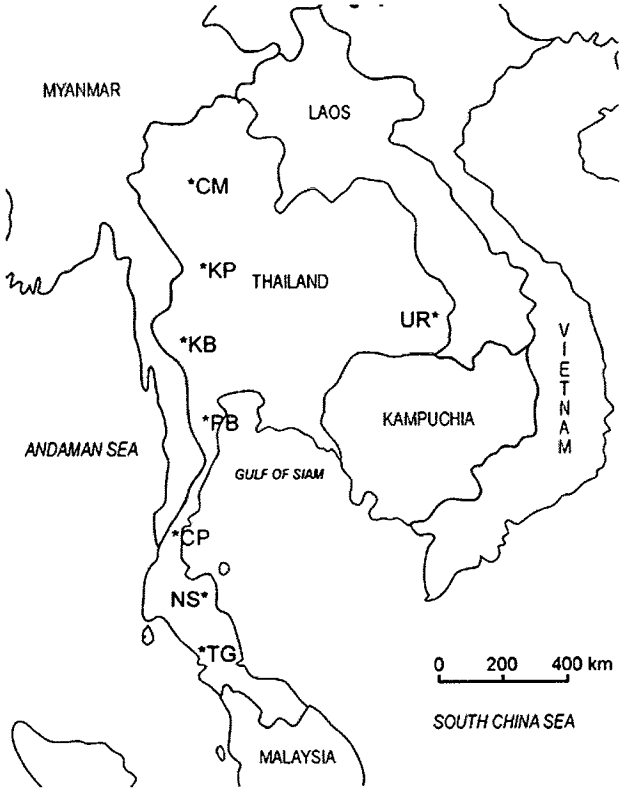

Fig. 1 Collection sites (*) for the *A. barbirostris/campestris* group in Thailand. *CM* Chiang Mai, *KP* Kamphaeng Phet, *KB* Kanchanaburi, *UR* Ubon Ratchathani, *PB* Phetchaburi, *CP* Chumphon, *NS* Nakhon Si Thammarat, *TG* Trang

Table 1 Localities where the *A. 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	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	*A. barbirostris* A1
	aCMA2	A (X_2,Y_1)	1,637	*A. barbirostris* A4
	hCME3	E (X_2,Y_5)	1,612	*A. campestris*-like
	hCME4	E (X_2,Y_5)	1,612	*A. campestris*-like
Kamphaeng Phet (16°28′ N, 99°31′ E)	aKPB1	B (X_2,Y_2)	1,612	*A. campestris*-like
Kanchanaburi (14°01′ N, 99°32′ E)	aKBA2	A (X_2,Y_1)	1,031	*A. barbirostris* A3
Ubon Ratchathani (15°15′ N, 104°52′ E)	aUBA7	A (X_2,Y_1)	1,822	*A. barbirostris* A1
Phetchaburi (13°09′ N, 100°04′ E)	aPBA3	A (X_2,Y_1)	1,678	*A. barbirostris* A2
Chumphon (10°29′ N, 99°11′ E)	aCPB4	B (X_1,Y_2)	1,822	*A. barbirostris* A1
Nakhon Si Thammarat (08°32′ N, 99°57′ E)	aNSD1	D (X_2,Y_4)	1,822	*A. barbirostris* A1
Trang (07°31′ N, 99°37′ E)	aTGA10	A (X_2,Y_1)	1,822	*A. barbirostris* A1

h human bait, *a* animal bait

amplified by polymerase chain reaction (PCR) using the following primers: ITS2A, 5′-TGTGAACTGCAGGACA CAT-3′ and ITS2B, 5′-TATGCTTAAATTCAGGGGGT-3′ for rDNA ITS2 (Beebe and Saul 1995). PCR and sequencing reactions were carried out as methods described by Saeung et al. (2007, 2008) and Suwannamit et al. (2009). To identify the mosquito species, the obtained sequence data were compared with the nucleotide sequences of the five sibling species deposited in the GenBank database (Saeung et al. 2007, 2008; Suwannamit et al. 2009).

Salivary gland dissection

Mosquitoes aged between 3 and 7 days after emergence were used. The female mosquitoes were cold anesthetized on ice before salivary gland dissection. Salivary glands of the mosquitoes were dissected in RNase-free phosphate-buffered saline [PBS; 10 mM Na_2SO_4, 145 mM NaCl (pH7.2)] using fine entomological needles under a stereoscopic microscope at ×4 magnification and transferred to a microcentrifuge tube with a small volume of PBS. Dissection of various regions of the female salivary glands was also performed. The medial lobes were cut at the junction of the medial and lateral lobes. The distal–lateral and proximal–lateral lobes were cut at the intermediate region separating the two lobes. The gland parts were immediately transferred to new tubes in order to avoid possible protein contamination between the different sections of the glands. The gland parts were placed in a small volume of PBS and stored at −80°C until use.

SDS–polyacrylamide gel electrophoresis

Salivary gland samples were thawed on ice and mixed in 1:2 (*v*/*v*) 1× sodium dodecyl sulfate (SDS) gel loading buffer [50 mM Tris–HCl (pH6.8), 100 mM DTT, 2% (*w*/*v*) SDS, 0.1% (*w*/*v*) bromphenol blue, 10% (*v*/*v*) glycerol]. Then the samples were heated for 5 min in a boiling water bath and loaded on 10% or 15% SDS–polyacrylamide gels. Protein molecular weight markers (Bio-Rad, USA) were applied in each gel. Following the electrophoresis, gels were Coomassie Brilliant Blue (CBB) stained. First, the gels were fixed in 50% methanol and 10% acetic acid for 30 min, then stained with 1% CBB in 10% methanol and 5% acetic acid for 2 h, and finally de-stained in 10% methanol and 5% acetic acid until dark protein bands were visible. Digital images of SDS–polyacrylamide gel electrophoresis (PAGE) CBB-stained gels were captured by scanning at 600 dpi using a color scanner. The images were stored and manipulated in PDF and TIFF formats using Photoshop™ 7.0 graphic software (Adobe Systems Inc., CA, USA).

In-gel digestion

Protein bands of interest were excised from the SDS–polyacrylamide gels using sterile surgical blades with aseptic technique. The gel pieces were subjected to in-gel digestion using an in-house method developed by Proteomics Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Thailand (Jaresitthikunchai et al. 2009). The gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM iodoacetamide in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for

5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid was added into the gels, and then the gels were incubated at room temperature for 10 min in a shaker. Peptides extracted were collected and pooled together in a new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at −80°C for further mass spectrometric analysis.

NanoLC-MS analysis and protein identification

The protein digest was injected into an Ultimate 3000 LC System (Dionex, USA) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker, Germany)) with electrospray at a flow rate of 300 nl/min to a nano-column (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id×150 mm). A solvent gradient (solvent A, 0.1% formic acid in water; solvent B, 80% 0.1% formic acid in 80% acetonitrile) was run for 40 min. Mascot from Matrix Science Ltd. (London, UK) was used to search all of the tandem mass spectra (Perkins et al. 1999). Protein identification was made on the basis of statistically significant Mowse score ($P<0.05$).

Results

Mosquito collection and identification of mosquito forms and species

Wild-caught, fully engorged females of the *A. 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 Fig. 2 and Table 1.

Analysis of salivary gland proteins of the mosquitoes by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and NanoLC-MS

Comparison of electrophoretic profiles of salivary glands obtained from female *A. barbirostris* species A1, A2, A3 and A4 and *A. 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, 32–37, 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 *A. 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 *A. gambiae* and *Anopheles freeborni* (Table 2).

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

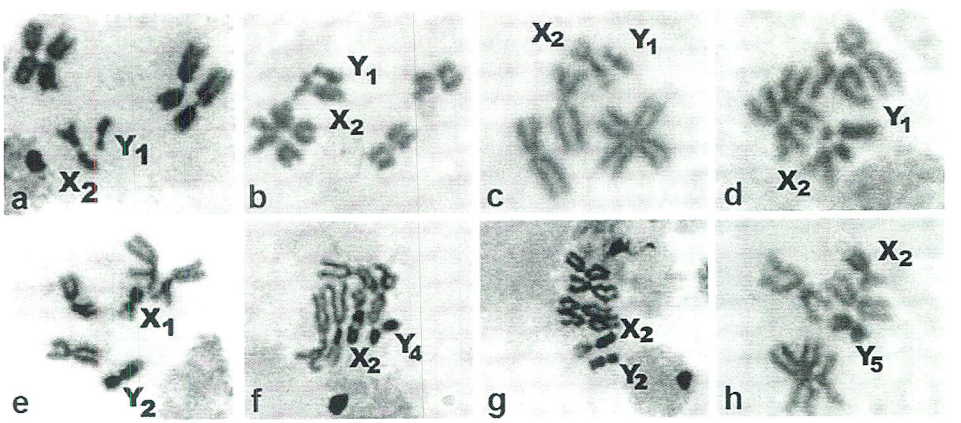

Fig. 2 Metaphase karyotypes of *A. barbirostris* complex. **a** Species A1, Form A (X_2, Y_1: Chiang Mai); **b** species A2, Form A (X_2, Y_1: Phetchaburi); **c** species A3, Form A (X_2, Y_1: Kanchaburi); **d** species A4, Form A (X_2, Y_1: Chiang Mai); **e** species A1, Form B (X_1, Y_2: Chumphon);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

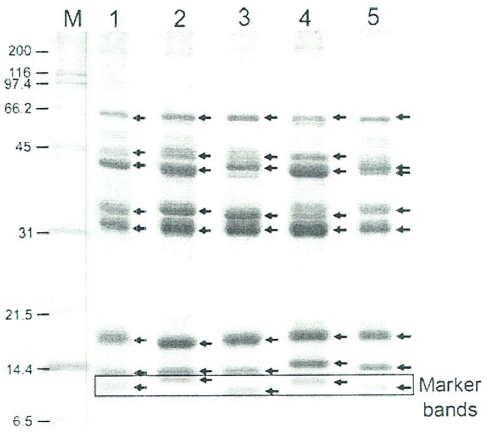

Fig. 3 Electrophoretic profiles of salivary glands obtained from female *A. barbirostris* complex. Proteins of one salivary gland pair were separated on 15% SDS–polyacrylamide gels and stained with Comassie blue. *Lane 1 A. barbirostris* species A1, *lane 2* species A2, *lane 3* species A3, *lane 4* species A4, *lane 5 A. 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

32–37 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, 32–34, 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).

Discussion

Thailand is an endemic area for malaria. A number of sibling species complexes of Anopheline mosquitoes are the human malaria vectors, for example, *Anopheles dirus* and *Anopheles minimus* (Rattanarithikul et al. 2006). Recently, *A. barbirostris* complex, i.e., *A. campestris*-like (Chiang Mai strain) and *A. 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. in preparation). The results showed that *A. campestris*-like (Forms B and E, Chiang Mai strain) was a high-potential vector whereas *A. barbirostris* species A1, A2, and A3 were low potential vectors. For *A. 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 *A. 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 *A. 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 *A. campestris*-like on 0.8% agarose gel.

In this study, we collected mosquitoes in the same and other localities for the *A. 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). *A. 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. *A. 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

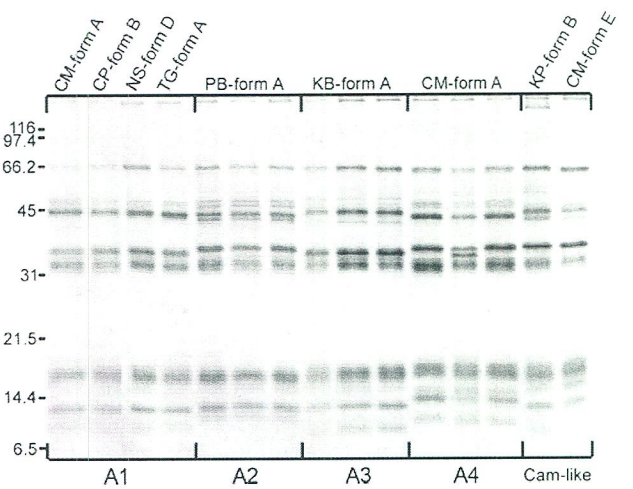

Fig. 4 Electrophoretic protein profiles of salivary glands obtained from female *A. barbirostris* species A1, A2, A3, and A4 and *A. campestris*-like. Proteins of one pair of salivary gland were separated on a 15% SDS–polyacrylamide gel and stained with Comassie blue. Mosquitoes from *CM* Chiang Mai, *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*	Description
A. barbirostris A1	gi|13537666	110	gSG6 protein [*A. gambiae*]
A. barbirostris A2	No match[a]	–	–
A. barbirostris A3	gi|229418592	119	gSG6 salivary protein [*A. freeborni*]
A. barbirostris A4	No match	–	–
A. campestris-like	gi|13537666	87	gSG6 protein [*A. gambiae*]

*$P<0.05$

[a] No match to *Anopheles* sequences

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 *A. 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 *A. 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 *A. 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

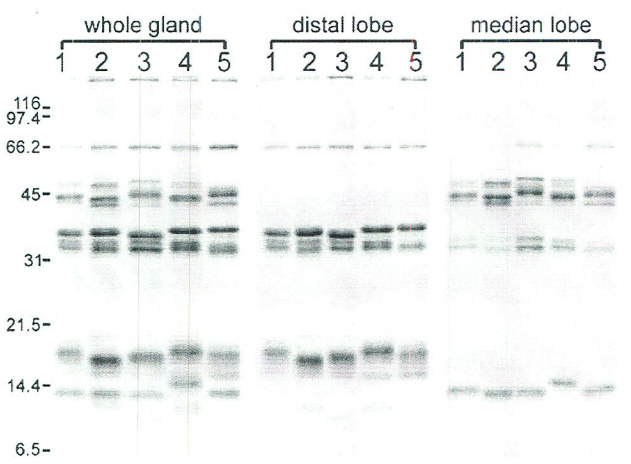

Fig. 5 Electrophoretic protein profiles of salivary glands obtained from female *A. barbirostris* species A1, A2, A3, and A4 and *A. campestris*-like. Proteins of one pair of whole salivary gland or distal lobe or median lobe were separated on a 15% SDS–polyacrylamide gel and stained with Comassie blue.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

relative mobility on SDS–polyacrylamide gels. But in *A. 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 *A. 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 *A. barbirostris* complex and *A. 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 *A. barbirostris* complex revealed that the protein of *A. barbirostris* species A1 and *A. campestris*-like matched a gSG6 protein of *A. gambiae* and of species A3 matched a gSG6 salivary protein of *A. 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 *A. gambiae* complex, i.e., *A. gambiae*, *Anopheles melas*, *Anopheles bwambae*, *Anopheles quadriannulatus A*, and *Anopheles arabiensis* (Lombardo et al. 2009). The gSG6 protein was originally identified in *A. 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 *A. 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 *A. freeborni* (subgenus *Anopheles*), the five species members of *A. gambiae* complex, *A. 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 *A. 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 *A. 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 *A. 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 *A. 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, *A. gambiae* (Brennan et al. 2000; Lombardo et al. 2009), *A. stephensi* (Suwan et al. 2002), and *Anopheles cracens* (formerly *A. dirus* B, Jariyapan et al. 2007). The protein profiles of the *A. barbirostris* complex in different salivary gland lobes showed similar distribution patterns to other *Anopheles* mosquitoes. Previous work with *A. aegypti*, *A. albopictus*, and *A. gambiae* indicated that the distal region of the lateral lobes synthesize and accumulate molecules that help in blood feeding. Examples are apyrase of *A. aegypti* and *A. albopictus* and gSG6 of *A. 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 *A. 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 *A. 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 *A. 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.

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

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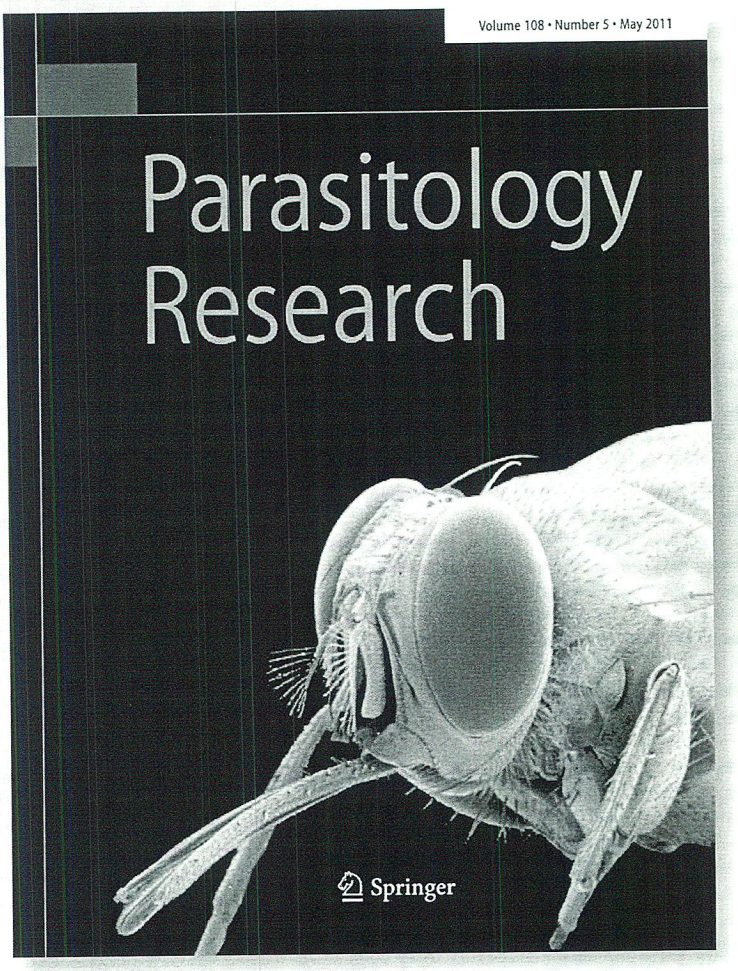

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ORIGINAL PAPER

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

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Abstract Salivary gland proteins of adult female *Anopheles barbirostris* species A2, a potential vector of *Plasmodium vivax* in Thailand, were analyzed using a proteomic approach (two-dimensional gel electrophoresis followed by nanoLC-MS). Two-dimensional gel electrophoresis revealed approximately 75 well-resolved spots on the reference gel. Most of the protein spots displayed relative molecular masses from 14 to 85 kDa and isoelectric points ranging from 3.9 to 10. The proteome profiles of *A. barbirostris* species A2 female salivary glands were affected by aging. The typical electrophoretic pattern of the female salivary glands was reached in 48 h post emergence, suggesting the maturation of salivary glands and saliva contents for blood feeding. 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, start to accumulate from emergence and gradually increase becoming predominant within 48 h. There are different salivary components expressed within each region of the female glands. The blood-feeding proteins were detected in the distal-lateral lobes and/or medial lobes. Proteins detected and/or identified by this approach could be tested in strategies developed to control pathogen and disease transmission. Moreover, the information of a 2D map of the female salivary gland could be used for comparison with other related species in the *A. barbirostris* complex to distinguish species members in the complex.

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Introduction

Salivary glands of adult female mosquitoes serve a dual function, assisting both blood and sugar meal feeding. The salivary proteins also play an important role in allergic responses in humans and animals and pathogen transmission. In anopheline mosquito vectors, prior to transmission, malaria parasites must invade the salivary glands and reside for some period of time in these organs (Ghosh et al. 2000; Ribeiro and Francischetti 2003).

Salivary glands of female *Anopheles* 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). Proximal regions of the lateral lobes produce enzymes involved in sugar feeding. Molecules

elated to blood feeding are synthesized by the medial and he distal regions of the lateral lobes (James 2003). Also, salivary gland invasion of malaria parasites is limited to he median lobe and distal regions of the lateral lobes (Rossignol et al. 1984; Pimenta et al. 1994). On the first day after emergence, females need to feed on sugar to meet the energy demands of basal metabolism and flight, and they need to feed on blood for egg development (Clements 1992).

In most of previous works on mosquito salivary gland proteins, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze and identify salivary gland proteins in each lobe (Suwan et al. 2002; Montero-Solis et al. 2004; Jariyapan et al. 2007, 2010). These papers showed that at least seven major protein bands were detected in the female salivary glands of each species, of which each morphological region contained different major proteins. Some protein bands might include more than one protein with different isoelectric points (pI). Therefore, the protein bands need to be analyzed with advanced techniques. Proteomics is a large-scale study of the gene expression at the protein level, which ultimately provides direct measurement of protein expression levels and insight into the activity state of all relevant proteins. Key elements of classical proteomics are the separation of proteins in a sample using two-dimensional gel electrophoresis (2-DE) and their subsequent identification by biological mass spectrometry (MS) (Nabby-Hansen et al. 2001). These techniques ensured good coverage of salivary gland proteins of various isoelectric points and molecular masses. Proteomic analyses of female salivary glands of *Anopheles gambiae* lead to the discovery of 57 novel proteins (Kalume et al. 2005). Several techniques were combined including SDS-PAGE, 2-DE, and liquid chromatography tandem mass spectrometry (LC-MS/MS) to describe *A. gambiae* salivary gland and saliva contents (Choumet et al. 2007). The study identified five saliva proteins and 122 more proteins from the salivary glands, including the first proteomic description for 89 of these salivary gland proteins. In the study, only 2-DE pattern of salivary gland proteins from mosquitoes aged 8 and 21 days after adult emergence were separated in the gels with pI 4–8 (Choumet et al. 2007).

Recently, *Anopheles barbirostris* species A2 has been proven to be a potential vector of *Plasmodium vivax* in Thailand (Thongsahuan et al. 2011). However, little is known regarding the salivary gland proteins of this mosquito species. Therefore, in this study, female salivary gland proteins of *A. barbirostris* species A2 were analyzed by 2-DE and mass spectrometry. We report, herewith, the expression of salivary gland proteins in mosquitoes aged varying from 0 to 60 h post emergence and the differential distribution of salivary components within the glands of female mosquitoes.

Materials and methods

Mosquito

A. barbirostris species A2 colonies (Saeung et al. 2008) were successfully maintained for many consecutive generations in an insectary at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand and were used in this study. The methods for rearing mosquitoes described by Choochote et al. (1983) and Kim et al. (2003) were used. The mosquitoes were reared and maintained in the insectary at 27±2 °C with 70±10 % relative humidity, and a photo-period of 12:12 (light/dark) h. Adult mosquitoes were given continuous access to a 10 % sucrose solution and fed on blood from immobilized mice when required. Mosquitoes aged between 0 and 60 h after emergence and fed only on sucrose solution were used in this study.

Salivary gland dissection

For age-dependent experiment, female mosquitoes were collected during 12 h intervals and dissected at 0–12, 24–36, and 48–60 h after emergence. The female mosquitoes were cold anesthetized on ice before salivary gland dissection. Salivary glands of the mosquitoes were dissected in phosphate-buffered saline (PBS; 10 mM Na_2SO_4, 145 mM NaCl (pH 7.2)) using fine entomological needles under a stereoscopic microscope at×4 magnification and transferred to a microcentrifuge tube with a small volume of PBS. For investigation of protein profiles in each salivary gland lobes, mosquitoes aged 48–60 h after emergence were used. Dissection of various portions of the female salivary glands was performed utilizing the method described by Jariyapan et al. (2010).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed using the 2D system (GE Healthcare, UK). A Micro BCA Protein Assay Kit (Pierce, USA) was used for quantification of proteins. The total salivary gland protein content of female mosquitoes at 48–60 h after emergence was on the average 1.25±0.05 μg/gland pair (70 gland pairs≈87 μg). Therefore, in each experimental sample, 70 pairs of female salivary glands were used. The salivary glands were extracted and desalted using a 2-D Clean-Up kit (GE Healthcare, UK). Each pellet sample was solubilized in a 125-μl sample solubilization solution (8 M urea, 50 mM dithiothreitol (DTT), 4 % CHAPS, 0.2 % 3/10 Bio-lyte Ampholyte, 0.002 % Bromophenol Blue) and then loaded on an IPG strip (pI 3–10, 7 cm, GE Healthcare, UK) to perform the first dimension isoelectric focusing separation. Following 13 h of

rehydration, the strips were focused using Ettan IPGphor III (GE Healthcare, UK) according to the manufacturer's instructions. The focused IPG strips were then incubated in 10 ml SDS equilibration buffer (6 M urea, 2 % SDS, 0.05 M Tris, pH 8.8, 30 % glycerol, 0.002 % Bromophenol Blue) containing 100 mg DTT for 15 min and for a further 15 min in 10 ml of equilibration buffer containing 250 mg iodoacetamide. The equilibrated strips were applied to the surface of vertical 15 % SDS-polyacrylamide gels and proteins separated in the second dimension using the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, USA). Protein molecular weight markers (Bio-Rad, USA) were applied in each gel.

Coomassie Brilliant Blue staining and gel image analysis

Following the electrophoresis, gels were Coomassie Brilliant Blue (CBB)-stained. First, the gels were fixed in 50 % methanol and 10 % acetic acid for 30 min, then stained with 1 % CBB in 10 % methanol and 5 % acetic acid for 2 h, and finally destained in 10 % methanol and 5 % acetic acid until dark protein bands were visible. The gels were scanned with the Image-scanner III (GE Healthcare, UK). A bioinformatics program (Image Master 2D Platinum, GE Healthcare, UK) was used to detect the number of spots in each gel and measure the molecular weight, the isoelectric point, and expression volume of each spot.

In-gel digestion

Protein spots of interest were excised from the 2-D gels using sterile surgical blades with aseptic technique. The gel pieces were subjected to in-gel digestion using an in-house method developed by Proteomics Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand (Jaresitthikunchai et al. 2009). The gel plugs were dehydrated with 100 % acetonitrile (ACN), reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM iodoacetamide in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100 % ACN for 5 min. To perform in-gel digestion of proteins, 10 μl of trypsin solution (10 ng/μl trypsin in 50 % ACN/10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 μl of 30 % ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37 °C for a few hours or overnight. To extract peptide digestion products, 30 μl of 50 % ACN in 0.1 % formic acid was added into the gels, and then the gels were incubated at room temperature for 10 min in a shaker. Peptides extracted were collected and pooled together in a new tube. The pool-extracted peptides were dried by vacuum centrifuge and kept at −80 °C for further mass spectrometric analysis.

NanoLC-MS analysis and protein identification

The protein digest was injected into an Ultimate 3000 LC System (Dionex, USA) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany) with electrospray at a flow rate of 300 nl/min to a nano-column (Acclaim PepMap 100 C18, 3 μm, 100A, 75 μm id×150 mm). A solvent gradient (solvent A: 0.1 % formic acid in water; solvent B: 80 % of 0.1 % formic acid in 80 % acetonitrile) was run for 40 min. Mascot from Matrix Science Ltd. (London, U.K.) was used to search all of the tandem mass spectra (Perkins et al. 1999). Protein identification was made on the basis of statistically significant Mowse score ($P<0.05$ or ≥ 30).

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 h after emergence. Two-dimensional gel electrophoresis protein profile of the female salivary glands at 0–12 h 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 h post emergence were also found in 24–36 and 48–60 h 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 h after emergence, respectively. As all major proteins were present from 48 h after emergence, the 2-DE profile of the female salivary glands at 48–60 h 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 Fig. 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 *A. barbirostris* species A2 is shown in Table 2. Expression

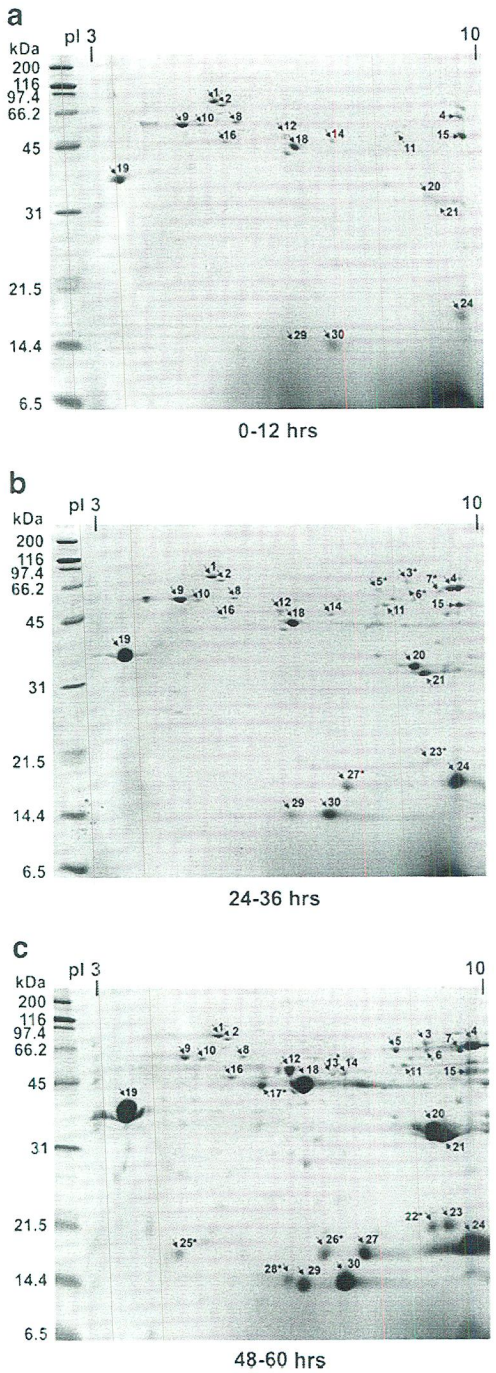

Fig. 1 Two-dimensional gel analysis of female salivary gland proteins of *A. barbirostris* species A2 mosquitoes according to age. Proteins were separated on Immobiline DryStrips 7 cm, pI 3–10. Separation in the second dimension was performed using 15 % SDS-PAGE. The gels were stained with Coomassie 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 h; **b:** 24–36 h; **c:** 48–60 h

volumes of 20 spots increased and two spots decreased within 60 h of mosquito development. From the 20 spots, ten spots increased in volume more than 1,000 fold. Among them, SN20, long form D7 protein (SN21), and D7-related 1 protein (SN24) had a high expression rate, respectively. The spots that decreased in volume were proteins with unknown function, SN8 and 9.

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 *A. barbirostris* species A2 are shown in Fig. 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 21 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), ten (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; Table 1).

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 *A. barbirostris* species A2 female salivary glands were affected by aging. The expression of most of salivary gland proteins in females varied from 0 to 60 h 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 0 and become predominant proteins within 48 h. The results suggested that the salivary gland proteins of female *A. barbirostris* species A2 reached the mature stage for feeding on blood within 48 h. Our results

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

Spot Number[a]	Accession number[b]	Protein description [Species]	Database MW/pI	Protein score[c]	No. of peptides/% coverage	First detection by 2-DE (h)[d]	Salivary gland region	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[e]					24	P	
4	gi|208657633	Putative 5′ nucleotidase/apyrase [*A. darlingi*]	63.527/ 8.72	114	3/4	0	D, M	Blood feeding
5	gi|157133637	Bifunctional purine biosynthesis protein [*A. 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 [*A. gambiae* str. PEST]	54.791/ 5.58	195	5/10	0	P, D, M	Unknown
9	gi|158300147	AGAP012407-PA [*A. 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 [*A. 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[e], M	
13	gi|58386650	AGAP008802-PA [*A. gambiae* str. PEST]	44.042/ 7.01	236	6/15	48	D, M	Unknown
14	gi|58390364	AGAP007827-PA [*A. 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[e], M	
18	gi|170041072	Conserved hypothetical protein [*C. quinquefasciatus*]	48.251/5.85	32	1/2	0	D[e], M	Unknown
19	gi|190576759	Anti-platelet protein [*A. gambiae*]	27.172/ 4.13	92	1/4	0	P[e], D	Blood feeding
20	NSH					0	P[e], D, M	
21	gi|114864717	Long form D7 salivary protein [*Anopheles funestus*]	36.720/ 8.45	47	2/5	0	P[e], D, M	Blood feeding
22	NSH					48	D	
23	gi|158289973	AGAP010375-PA [*A. gambiae* str. PEST]	17.651/ 10.43	73	3/11	24	D	Unknown
24	gi|4538887	D7-related 1 protein [*A. 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	

NSH Not significant hit, *P* Proximal-lateral lobe, *D* Distal-lateral lobe, *M* Median lobe

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

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

[e] Low amount of expression

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

Spot Number[a]	MW[b]	pI[c]	0–12 h[d]		24–36 h[e]		48–60 h[f]	
			ANV	Index[g]	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

ANV average normalization volume of protein spot

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

[b]MW: observed molecular mass

[c]pI: observed isoelectric point

[d]0–12 h post emergence

[e]24–36 h post emergence

[f]48–60 h post emergence

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

are consistent with previous studies in *Anopheles darlingi*, *Anopheles stephensi*, *Culex pipiens* and *Culex 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 *A. darlingi* mosquitoes with ages varying from 1 to 10 days after adult emergence. The protein pattern of female *A. darlingi* salivary glands does not vary qualitatively during the first 10 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 *A. 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 *Aedes 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 *A. aegypti*, the salivary apyrase was identified as a member of the 5′ nucleotidase family (Champagne et al. 1995). Recently, apyrase of *Aedes 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 4 days after adult emergence and remain high after a blood meal. The biochemical analyses of the salivary glands of female *A. darlingi* (Marinotti et al. 1996) and *Anopheles dirus* B (Jariyapan et al. 2007) revealed the presence of apyrase

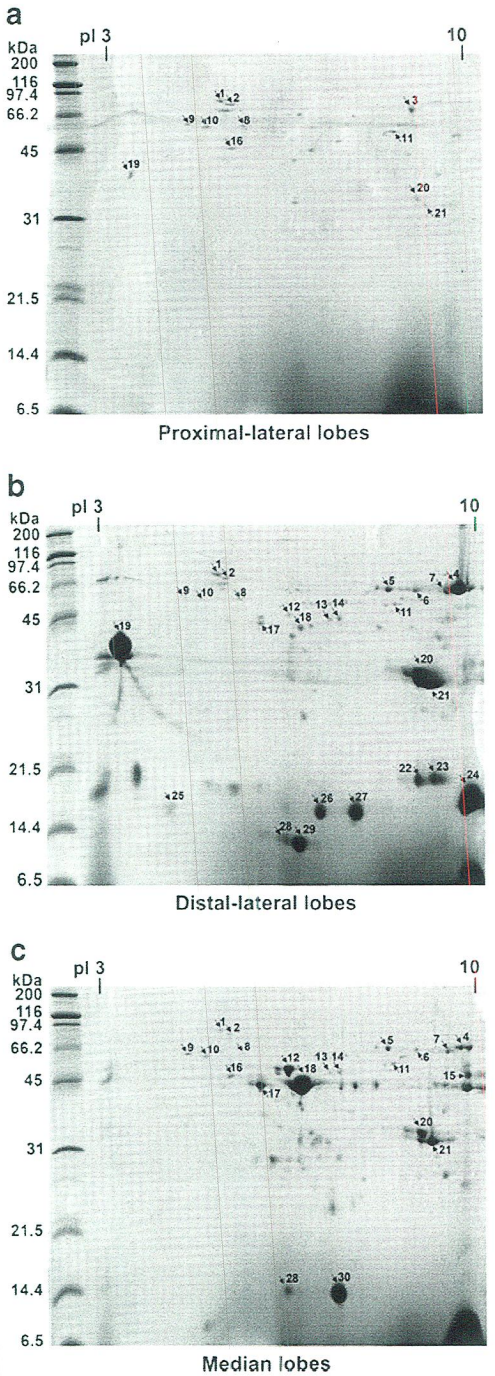

Fig. 2 Two-dimensional gel analysis of proteins expressed in the different regions of *A. barbirostris* species A2 female salivary glands. Proteins were separated on Immobiline DryStrips 7 cm, pI 3–10. Separation in the second dimension was performed using 15 % SDS-PAGE. The gels were stained with Coomassie 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 h; **b** distal-lateral lobes; **c** median lobe

activity in the distal region of the lateral lobes (~66 %), median lobe (~23 %), and proximal region of the lateral lobes (~11 %). In this study, *A. 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 *A. 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 *A. 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, glutamic acid, and aspartic acid 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 *A. 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 *A. 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 insect (James et al. 1991; Arca et al. 1999; 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. 2006). 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 *A. 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 *A. 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 *A. 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 *A. 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 *A. barbirostris* species A2 salivary glands correlates with a previous study in *A. stephensi* (Suwan et al. 2002). For the D7 short form, it was expressed only in the distal-lateral lobes as detected in *A. 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 *A. barbirostris* species A2. This result correlates with a study in *A. gambiae* that gSG6 was expressed only in distal-lateral lobes (Lombardo et al. 2009). The gSG6 protein was first identified in the female *A. gambiae* mosquito (Lanfrancotti et al. 2002). It was conserved in five species members of the *A. gambiae* complex, i.e., *A. 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 *A. 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 *A. gambiae* salivary glands was designed as a specific salivary sequence of malaria vector species. It was shown that the quantification of human antibody 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 *A. barbirostris* species A2. They were found from 0 h after emergence and their amount increased gradually within 48 h. F0/F1-type ATP synthase was detected in 2-DE maps of female *A. aegypti* salivary glands (Ribeiro et al. 2007) and in 1-D gel of *A. gambiae* female salivary glands (Kalume et al. 2005). 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 *A. 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 *A. 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 h 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 *A. aegypti* (Thangamani and Wikel 2009) and *A. gambiae* (Kalume et al. 2005; Wang et al. 2010).

One spot that matched a bifunctional purine biosynthesis protein of *A. aegypti* was detected from 24-h post emergence. However, the function of the protein in mosquitoes has not been reported. In humans, 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 5-aminoimidazole-4-carboxamide-ribonucleotide (AICAR) transformylase, which catalyzes the formylation of AICAR by N-10-formyl-tetrahydrofolate to produce formyl-AICAR (FAICAR) and THF. ATIC also acts as an inosine monophosphate (IMP) cyclohydrolase, 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 *A. gambiae* and *C. quinquefasciatus*, respectively. Two of them, AGAP007393-PB [*A. gambiae* str. PEST] (SN8) and AGAP012407-PA [*A. 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 example, 1-DE followed by LC-MS/MS, in-solution followed by LC-MS/MS, and iTRAQ labeling. These approaches may help to identify more proteins in the salivary glands as the previous studies in *A. gambiae* (Kalume et al. 2005; Choumet et al. 2007).

Recently, Juhn et al. (2011) reported the hybridization in situ patterns of 30 genes expressed in the salivary glands of adult female *A. 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 involved 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 *A. 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 accumulating from 48 h 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 *A. barbirostris* species A2 were analyzed for the first time using a proteomic approach (2-DE followed by nanoLC-MS). The proteome profiles of *A. barbirostris* species A2 female salivary glands were affected by aging. As feeding on blood of mosquitoes depends on maturation of salivary glands and saliva contents, the salivary glands of female *A. barbirostris* species A2 are mature from 48 h post emergence. Proteins involved in blood feeding started to accumulate from 0 h after emergence and gradually increased and became predominant within 48 h. 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 *A. barbirostris* species A2 female salivary gland that could be used to compare with other related species in the *A. barbirostris* complex (Jariyapan et al. 2010) for identification of different proteins that could be used to distinguish mosquito species members in the complex.

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Sor-suwan S, **Jariyapan N**, Roytrakul S, Paemanee A, Saeung A, Thongsahuan S, Phattanawiboon B , Bates PA, Poovorawan Y, Choochote W. (2012) Salivary gland proteome of the human malaria vector, *Anopheles campestris*-like (Diptera: Culicidae). Parasitol Res (submitted).

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Salivary Gland Proteome of the Human Malaria Vector, Anopheles campestris-like (Diptera: Culicidae)

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Salivary Gland Proteome of the Human Malaria Vector, *Anopheles campestris*-like (Diptera: Culicidae)

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Abstract *Anopheles campestris*-like is proven to be a high-potential vector of *Plasmodium vivax* in Thailand. In this study, *An. campestris*-like salivary gland proteins were determined and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis and nano-liquid chromatography-mass spectrometry. The total amount of salivary gland proteins in the mosquitoes aged 3-5 days was approximately 0.1 ± 0.05 µg/male and 1.38 ± 0.01 µg/female. SDS-PAGE analysis revealed at least 12 major proteins found in the female salivary glands and each morphological region of the female glands contained different major proteins. Two-dimensional gel electrophoresis showed approximately 20 major and several minor protein spots displaying relative molecular masses from 10-72 kilodaltons with electric points ranging from 3.9-10. At least 15 glycoproteins were detected in 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. 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 (DL) and/or medial lobes (ML) of the female glands. The major spots related to housekeeping proteins from other arthropod species including *Culex quinquefasciatus* serine/threonine-protein kinase rio3 expressed in both male and female glands, *Ixodes scapularis* putative sil1 expressed in DL and ML, and *I. scapularis* putative cyclophilin A expressed in DL. These results provide information for further study on the salivary gland proteins of *An. campestris*-like that are involved in hematophagy and disease transmission.

Keywords *Anopheles*, mosquito, salivary gland, proteome, mass spectrometry, 2-DE

Introduction

Malaria is a parasitic disease caused by protozoa in the genus *Plasmodium*. It affects 200 million people worldwide causing 1.5 to 2.7 million deaths per year, of the 300-500 million clinical cases annually (WHO 2008). *Anopheles* mosquitoes are the exclusive vectors of human malaria. The salivary glands are of interest in the *Anopheles* mosquito because malaria sporozoites must invade the mosquito salivary glands in order to be transmitted to a human host (Ghosh et al. 2000; Ribeiro and Francischetti 2003). The salivary glands of adult mosquitoes are sexually dimorphic and it is clear that their structural and functional differences enable females to engage successfully in hematophagy. The salivary glands of adult female *Anopheles* mosquitoes have a distinctive tri-lobed structure consisting of a single medial lobe and two lateral lobes with distinct proximal and distal portions (Wright 1969; Moreira-Ferro et al. 1999; Jariyapan et al. 2007). When *Plasmodium* sporozoites invade the salivary glands, the mosquito becomes infectious. It is known that sporozoites only invade the distal portion of lateral lobes and the medial lobes and that recombinant circumsporozoite (CS) protein binds specifically to the *Anopheles stephensi* salivary glands, particularly to the medial and distal-lateral lobes of the gland (Sidjanski et al. 1997). The salivary glands contain a variety of physiologically and biochemically active molecules that involve food ingestion and digestion. Mosquito saliva is vital for successful blood feeding because it contains anti-coagulant, anti-inflammatory, and immunosuppressive factors. In addition, saliva proteins are antigenic and immunogenic, involving immunoglobulin E, immunoglobulin G and T-lymphocyte mediated hyposensitivity response in the vertebrate host (Ribeiro and Arca 2009; Ribeiro et al. 2010).

Recently, *Anopheles campestris*-like (Chiang Mai strain), a member of the *Anopheles barbirostris* complex in Thailand, was experimentally infected with *Plasmodium vivax* and *Plasmodium falciparum*. The results revealed that *An. campestris*-like was a high-potential vector for *P. vivax* with 66.67% sporozoite rates when compared to 90% sporozoite rates recovered from *Anopheles cracens*. But it was a refractory vector for *P. falciparum* (Thongsahuan et al. 2011). Preliminary analysis of female salivary gland proteins of *An. barbirostris* complexes were performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and nano-liquid chromatography-mass spectrometry (nanoLC-MS) (Jariyapan et al. 2010). For *An. campestris*-like, at least eight major and several minor protein bands were detected in the glands. The nanoLC-MS analysis revealed that a major protein band matched with a protein involving in blood feeding, gSG6, of *Anopheles gambiae* and *Anopheles freeborni*. No more proteins were reported as not all proteins could be identified by the techniques (Jariyapan et al. 2010). Because, some protein bands might be mixed polypeptides which have the same molecular mass but differ in isoelectric points (pI). Therefore, advanced techniques are required to analyze the proteins.

Two-dimensional gel electrophoresis (2-DE) is a powerful and high throughput tool for describing the changes in protein expression and modification, which involves separation of cellular proteins according to their isoelectric points and relative molecular masses. The separation of proteins in a sample using 2-DE and their subsequent identification by biological mass spectrometry (MS) are key elements of classical proteomics, which enables investigating the gene expression at the protein level (Nabby-Hansen et al. 2001). These techniques were used to study the salivary gland proteomes of several arthropods, for examples, *An. gambiae* (Choumet et al. 2007), *Aedes aegypti* (Wasinpiyamongkol et al. 2010), *Culicoides nubeculosus* (Langner et al. 2007; Wilson et al. 2008; Russell et al. 2009), *Rhipicephalus haemaphysaloides* (Xiang et al. 2009).

In this study, total amounts of salivary gland proteins of both the male and female *An. campestris*-like mosquitoes were determined. Also, SDS-PAGE and 2-DE coupled with nanoLC-MS were applied to identify major proteins differentially expressed in the salivary glands of males and each salivary gland lobes of females. In addition, glycoproteins in the female glands were identified. This new data could lead to further study on proteins involved in blood feeding and pathogen transmission to vertebrate hosts.

Materials and methods

Mosquito

An. campestris-like colonies (Thongsahuan et al. 2009) were successfully maintained for many consecutive generations in an insectary at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand and were used in this study. The methods for rearing mosquitoes described by Choochote et al. (1983) and Kim et al. (2003) were used. The mosquitoes were reared and maintained in the insectary at 27 ± 2 °C with 70 ± 10 % relative humidity, and a photo-period of 12:12 (light/dark) h. Adult mosquitoes were given continuous access to a 10 % sucrose solution and fed on blood from immobilized mice when required. Mosquitoes aged between 3 and 5 days after emergence and fed on sucrose solution were used in this study.

Salivary gland dissection

Salivary gland dissection was performed utilizing the method described by Jariyapan et al. (2010). Adult mosquitoes between 3-5 days of age were cold anaesthetized on ice before salivary gland dissection. Salivary glands of the mosquitoes were dissected in phosphate-buffered saline [PBS; 10 mM Na_2SO_4, 145 mM NaCl (pH 7.2)] using fine entomological needles under a stereoscopic microscope at 4X magnification and transferred to a microcentrifuge tube with a small volume of PBS. The samples were then kept at -80 °C until use. Dissection of the various regions of the female salivary glands was performed. The medial lobes were cut at the junction of the medial lobes and the lateral lobes. The distal-lateral and proximal-lateral lobes were cut at the intermediate region separating the two lobes. The gland parts were immediately removed to separate tubes to avoid possible protein contamination between the different sections of the glands. The gland parts were placed in a small volume of PBS and stored at -80 °C until use.

SDS-PAGE

Salivary gland samples were thawed on ice and mixed in 1:2 (v/v) 1XSDS gel loading buffer (50mM Tris-HCl, pH 6.8, 100 mM DTT, 2 % SDS, 0.1 % Bromphenol blue, 10 % glycerol). Then, the samples were heated for 5 min in a boiling water bath and loaded on 15% SDS polyacrylamide gels. Molecular weight markers (Bio-Rad Laboratories; Hercules, CA) were applied in each gel.

Two-dimensional gel electrophoresis

2-DE was performed using the 2D system (GE Healthcare, UK). The protein concentration was determined by the Bradford method using Bio-Rad protein assay (Bio-Rad Laboratories; Hercules, CA). In each female sample, 70 pairs of female salivary glands (≈ 90 μg of total proteins) were used. For the male sample, 200 pairs of salivary glands (≈ 20 μg of total proteins) were used. The salivary glands were extracted and desalted using a 2-D Clean-Up kit (GE Healthcare, UK). Each pellet sample was solubilized in a 125 μl sample solubilization solution (8 M urea, 50 mM DTT, 4 % CHAPS, 0.2 % 3/10 Bio-lyte Ampholyte, 0.002 % Bromophenol Blue) and then loaded on an IPG strip (pI 3–10, 7 cm, GE Healthcare, UK) to perform the first dimension isoelectric focusing (IEF) separation. Following 13 h rehydration, the strips were focused using Ettan IPGphor III (GE Healthcare, UK) according to the manufacturer's instruction. The focused IPG strips were then incubated in 10 ml SDS equilibration buffer (6 M urea, 2 % SDS, 0.05 M Tris, pH 8.8, 30 % glycerol, 0.002 % Bromophenol blue) containing 100 mg DTT for 15 min and for a further 15 min in 10 ml equilibration buffer containing 250 mg iodoacetamide. The equilibrated strips were applied to the surface of vertical 15 % SDS-polyacrylamide gels and proteins separated in the second dimension using the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad Laboratories; Hercules, CA). Protein molecular weight markers (Bio-Rad Laboratories; Hercules, CA) were applied in each gel.

Coomassie Brilliant Blue (CBB) staining, glycoprotein staining, and gel image analysis

Following the electrophoresis, the gels were CBB stained. First, the gels were fixed in 50 % methanol and 10 % acetic acid for 30 min, then stained with 1 % CBB in 10 % methanol and 5 % acetic acid for 2 h, and finally de-stained in 10 % methanol and 5 % acetic acid until dark protein bands were visible. The gels were scanned with the Imagescanner III (GE Healthcare, UK). For glycoproteins, the gels were stained with Pro-Q Emerald 300 glycoprotein stain (Invitrogen, OR) according to the manufacturer's instruction. A bioinformatics program (Image Master 2D Platinum, GE Healthcare, UK) was used to detect the number of spots in each gel, measure the molecular weight and the isoelectric point of each spot, and determine volume of each spot.

Protein quantification and statistical analysis

A Micro BCA protein assay kit (Pierce, Rockford, IL) was used for the quantification of proteins according to the manufacturer's instruction. The mean and standard error of the mean (SEM) were calculated for the total proteins of each salivary gland pair (n=25).

In-gel digestion

Protein spots of interest were excised from the 2-DE gels using sterile surgical blades with aseptic technique. The gel pieces were subjected to in-gel digestion using an in-house method developed by Proteomics Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand (Jaresitthikunchai et al. 2009). The gel plugs were dehydrated with 100b% acetonitrile (ACN), reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM

iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100 % ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50 % ACN/10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 µl of 30 % ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37 °C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50 % ACN in 0.1 % formic acid (FA) was added into the gels, and then the gels were incubated at room temperature for 10 min in a shaker. The extracted peptides were collected and pooled together in a new tube. The extracted peptide pools were dried by vacuum centrifuge and kept at -80 °C for further mass spectrometric analysis.

NanoLC-MS analysis and protein identification

The protein digest was injected into an Ultimate 3000 LC System (Dionex, Sunnyvale, CA) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany) with electrospray at a flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id x 150 mm). A solvent gradient (solvent A: 0.1 % formic acid in water; solvent B: 80 % of 0.1 % formic acid in 80 % acetonitrile) was run for 40 min. Mascot from Matrix Science Ltd. (London, UK) was used to search all of the tandem mass spectra (Perkins et al. 1999). The data were sent to the National Center for Biotechnology nonredundant (NCBInr) protein database. The search was performed taking Other Metazoa as taxonomy. The other search parameters were enzyme of specificity strict trypsin; one missed cleavage; fixed modifications of Carbamidomethyl (C); oxidation (Met); peptide tolerance of 100 ppm; Fragment Mass Tolerance of ± 0.5 Da; peptide change of 1+; and monoisotopic. Protein identification was made on the basis of statistically significant Mowse score ($P < 0.05$ or ≥ 30).

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).

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. 2005; 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. 2005; 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.

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Figure legends

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

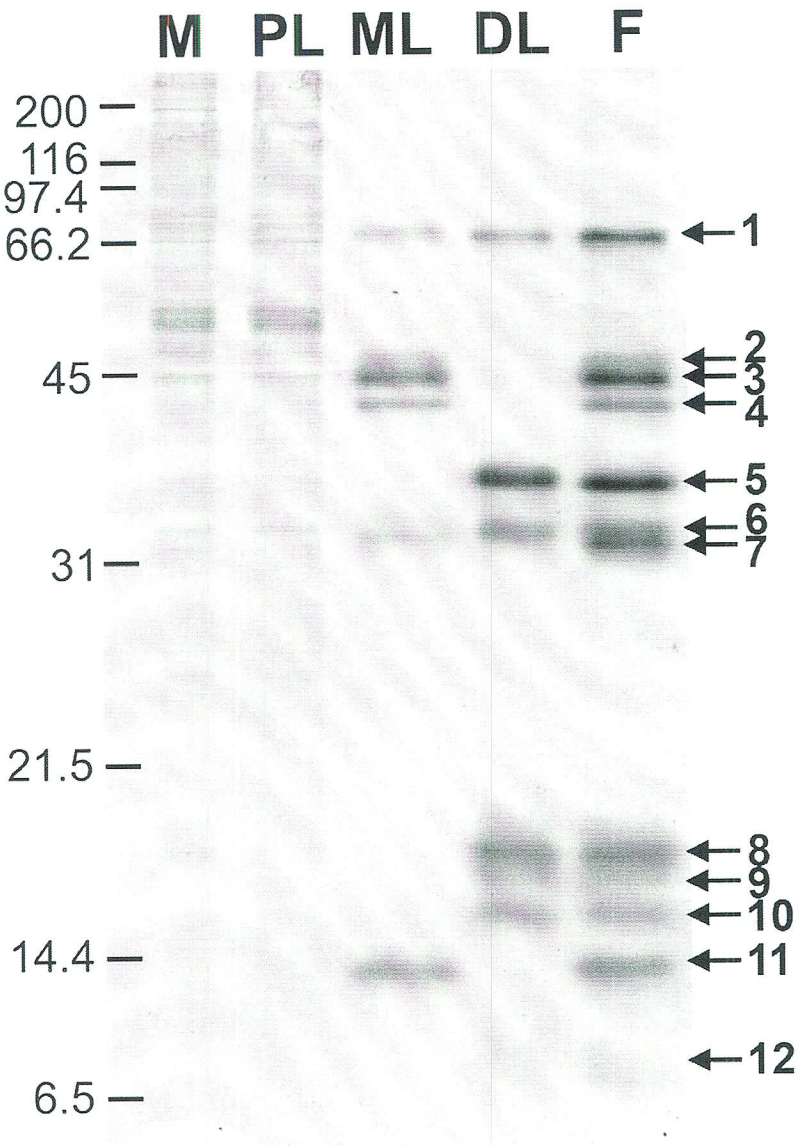
Fig. 3 Two-dimensional gel analysis of proteins expressed in male and different regions of female *An. campestris*-like salivary glands. Proteins were separated on Immobiline DryStrips 7 cm, pI 3-10. Separation in the second dimension was performed using 15% SDS-PAGE. The gels were stained with CBB. 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

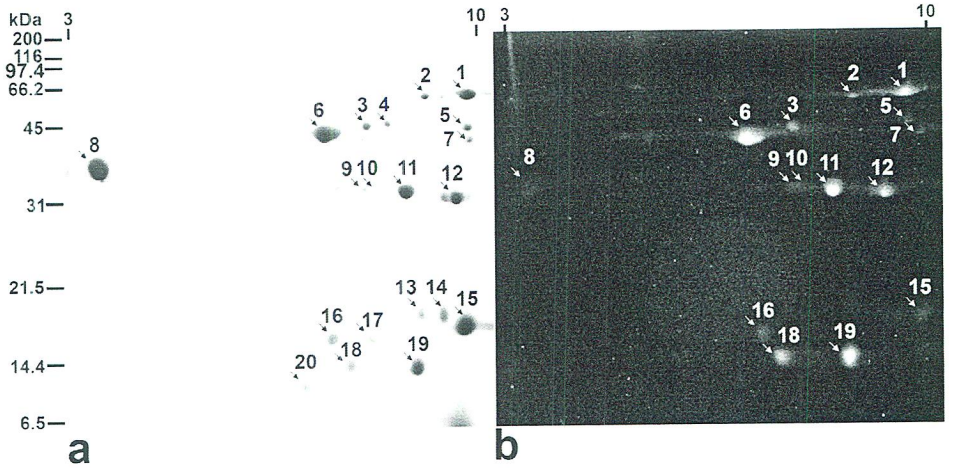
SN[a]	Accession number[b]	Protein description [species]	Protein score[d]	No. of peptides/ % coverage	Database Mw/pI	Observed Mw/pI	Exp. Pattern[e]	Glycoprotein	Classification
1	gi|4582524	apyrase [*Anopheles gambiae*]	31	1/1	62.1/8.9	68/9.8	M[f], PL[f], ML, DL	yes	Blood feeding
2	gi|208657633	putative 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.*	55	1/6	19.1/9.2	20/7.9	DL	no	Blood feeding

		gambiae]							
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

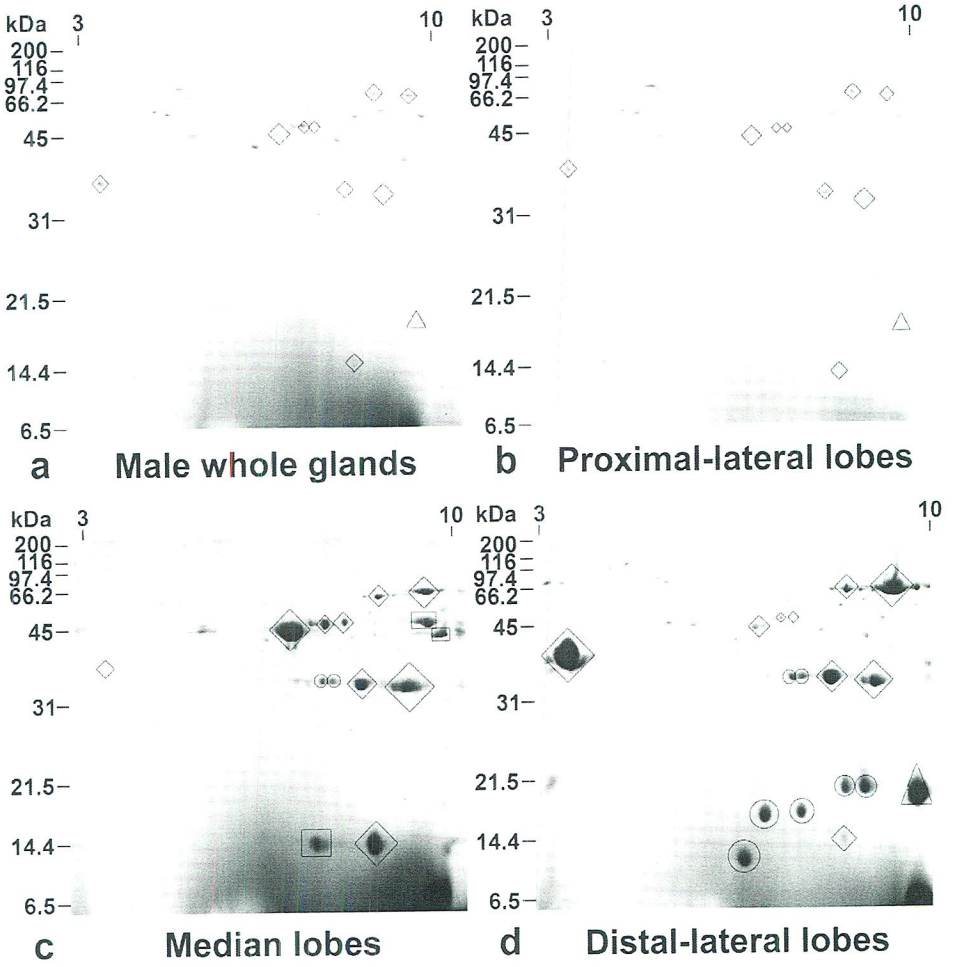
[a]Spot number refers to those shown in Fig. 2a
[b]Accession number of the best hit of proteins from mosquitoes and/or arthropod species
[c]NSH = not significant hit
[d]$P < 0.05$ or MASCOT score ≥30
[e]Expression pattern in male and different regions of female salivary glands: M, male; PL, proximal-lateral lobe; DL, distal-lateral lobe; ML, medial lobe
[f]Low amount of expression
[g]Low intensity



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
70x99mm (300 x 300 DPI)



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
119x57mm (300 x 300 DPI)



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, ρ = a major spot found in male, proximal-lateral and distal-lateral lobes, ƽ = a major spot found in medial and distal-lateral lobes, ↓ = a major spot found in male and all female lobes
140x140mm (300 x 300 DPI)

เอกสารแนบหมายเลข 4

ผลงานนำเสนอแบบโปสเตอร์ เรื่อง "Analysis of salivary gland proteins in *Anopheles barbirostris* complex (Diptera: Culicidae) in Thailand" ในการประชุมวิชาการ "การประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 9" ที่ โรงแรมฮอลิเดย์อินน์ รีสอรทรีเจนท์ บีช ชะอำ จ. เพชรบุรี วันที่ 15-17 ตุลาคม พ.ศ. 2552

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

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Introduction and Objective

Analyses of salivary gland proteins (proteomes) and/or genes (transcriptomes) were performed in a number of *Anopheles* mosquitoes. These studies led to the discovery of several novel protein families providing some clues on the evolution of blood feeding and revealing the complexity of mosquito salivary secretions. Calvo et al. (2004) compared *Anopheles darlingi* (subgenus *Nyssorhynchus*) and *A. gambiae* (subgenus *Celia*) salivary gland genes belonging to the secreted and housekeeping categories and concluded that the genes encoding secreted products are rapidly evolving in comparison with the housekeeping genes. Valenzuela et al. (2003) found similar results when the salivary gland transcriptomes of *A. stephensi* and *A. gambiae* were compared. These results support the hypothesis that secreted genes may be good markers for assessing phylogeny among closely related species, as has been demonstrated with triatomine bugs (Soares et al. 2000).

The *A. barbirostris/campestris* group was incriminated as potentially natural vectors of *P. vivax* in the Aranyaprathet district, Sa Kaeo province, eastern Thailand (Limrat et al. 2001; Apiwathnasorn et al. 2002). In addition, they were also considered as vectors that played an important role in increasing cases of *P. vivax* infection in Thailand (Sattabongkot et al. 2004). Recently, at least 5 sibling species members, namely, *An. campestris*-like and *A. barbirostris* species A1, A2, A3 and A4 were discovered within the taxon *A. barbirostris* (Saeung et al. 2007, 2008; Suwannamit et al. 2009; Thongsahuan et al. 2009). In this study, therefore, electrophoretic protein profiles of female salivary glands of each forms and sibling species in the taxon *A. barbirostris* were analyzed.

Materials and methods

1. Mosquito collection and isoline colonization
Wild-caught, fully engorged females of the *A. barbirostris* complex were collected using both human-baited and animal-baited traps in 8 provinces in Thailand (Table 1). Isoline colonization was performed using the techniques by Choochote et al. (1983) and Kim et al. (2003).
2. Mitotic karyotype
Metaphase chromosomes were prepared using the method of Baimai et al. (1995).
3. Identification of mosquito species using rDNA ITS2 region as a molecular marker
(Saeung et al. 2007, 2008; Suwannamit et al. 2009).
4. Salivary gland dissection (Jariyapan et al. 2007)
5. One-dimensional gel electrophoresis (Jariyapan et al. 2007)
6. In-gel digestion and NanoLC-MS analysis (Perkins et al. 1999; Jaresitthikunchai et al. 2009).

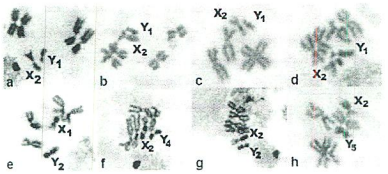

Fig. 1 Metaphase karyotypes of *A. barbirostris* complex. **(a)** species A1, Form A (X2, Y1: Chiang Mai); **(b)** species A2, Form A (X2, Y1: Phetchaburi); **(c)** species A3, Form A (X2, Y1: Kanchaburi); **(d)** species A4, Form A (X2, Y1: Chiang Mai); **(e)** species A1, Form B (X1, Y2: Chumphon); **(f)** species A1, Form D (X2, Y4: Nakhon Si Thammarat); **(g)** *A. campestris*-like, Form B (X2, Y2: Kamphaeng Phet); **(h)** *A. campestris*-like, Form E (X2, Y5: Chiang Mai).

Table 1 showing the localities where the *A. 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*	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)	1,622	*An. barbirostris*-A1

* h – human bait, a – animal bait

Acknowledgments

This work was financially supported by the Thailand Research Fund (RMU 5180011).

Results

1. Mosquito collection and identification of mosquito forms and species

Results of mosquito forms and species are shown in Figure 1 and Table 1.

2. Analysis of salivary gland proteins of the mosquitoes by SDS-PAGE and nanoLC-MS :Figure 2 and 3.

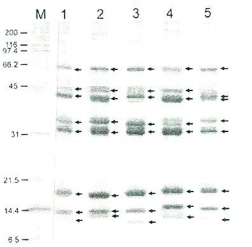

Fig. 2 Electrophoretic profiles of salivary glands obtained from female *A. barbirostris* complex. Proteins of one salivary gland pair were separated on 15% SDS-polyacrylamide gels and stained with Comassie blue. Lane 1, *An. barbirostris* species A1; lane 2, species A2; lane 3, species A3; lane 4, species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

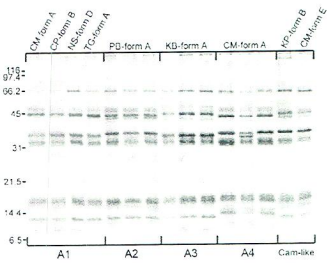

Fig. 3 Electrophoretic profiles of salivary glands obtained from female *An. barbirostris* A1, *An. barbirostris* A2, *An. barbirostris* A3, *An. barbirostris* A4, and *An. campestris*-like. Proteins of one pair of salivary gland were separated on a 15% SDS-polyacrylamide gel and stained with Comassie blue. Chiang Mai (CM), Kamphaeng Phet (KP), Chumphon (CP) and Nakhon Si Thammarat (NS). (B) Mosquitoes from Ubon Ratchathani (UR) and Trang (TG). Molecular mass markers are shown on the left in kilodalton.

3. Distribution of female salivary gland proteins

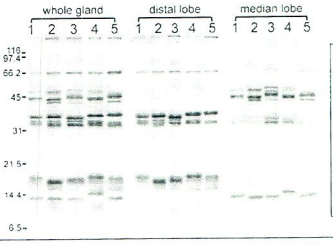

Fig. 4 Electrophoretic profiles of salivary glands obtained from female *An. barbirostris* A1, *An. barbirostris* A2, *An. barbirostris* A3, *An. barbirostris* A4, and *An. campestris*-like. Proteins of one pair of whole salivary gland or distal lobe or median lobe were separated on a 15% SDS-polyacrylamide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

Discussion and Conclusion

As morphologically indistinguishable, incorrect identification of individual members in the species complex may result in failure to distinguish between a vector and non-vector species, and lead to the complication and/or unsuccessful formation of control strategies for *A. barbirostris* species complex.

Our initial finding revealed the differences of the electrophoretical protein profiles of the female salivary glands of these *A. barbirostris* complex. Moreover, the protein band with the molecular mass of about 10-13 kDa range in each species showed remarkably different relative mobility on SDS-PAGE. LC-MS analysis revealed that the proteins match gSG6 protein family of other anopheles mosquitoes, suggesting that they might be used as a marker for assessing phylogeny among these five closely related species.

These results would be useful for construction of an additional tool to distinguish the five sibling species and lead to further study on evolution of the *A. barbirostris* complex in blood feeding and pathogen transmission.

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เอกสารแนบหมายเลข 5

ผลงานนำเสนอแบบโปสเตอร์ เรื่อง "Salivary gland proteins of the human malaria vector, *Anopheles campestris*-like (Diptera: Culicidae)" ในการประชุมวิชาการ "RGJ seminar series LXXIV - From basic biomedical research to sustainable development" ที่ โรงแรมศิรินาถการ์เด้น จ.เชียงใหม่ วันที่ 16 กันยายน พ.ศ. 2553

Salivary Gland Proteins of the Human Malaria Vector, *Anopheles campestris*-like (Diptera: Culicidae)

Sriwatapron Sor-suwan,[a] Visut Baimai,[b] Wej Choochote,[a] Sittiruk Roytrakul,[c] Atiporn Saeung,[a] Sorawat Thongsahuan,[a] Krissana Ta-ai,[a] Yong Poovorawan,[d] Narissara Jariyapan[a]

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Introduction and Objective

Salivary glands are of interest in anopheline mosquitoes because transmission of malaria to vertebrate hosts depends on the ability of *Plasmodium* sporozoites to invade the salivary glands of female vectors and malaria parasites must reside for some period of time in these organs. Salivary glands and the saliva of mosquito vectors have also attracted considerable attention because of their role in blood feeding, and involvement in allergic responses in humans and animals.

Recently, *An. campestris*-like has been proven to be a high-potential vector of *P. vivax* in Thailand. However, little is known regarding the salivary gland proteins of this mosquito species. Therefore, salivary gland proteins *An. campestris*-like were determined and analyzed in this study.

Materials and methods

1. Mosquito

An. campestris-like Form E (Chiang Mai strain, iHCE6) (Thongsahuan et al, 2009) were successfully maintained for many consecutive generations in an insectary at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand and were used in this study. The methods for rearing mosquitoes described by Choochote et al (1983) and Kim et al (2003) were used.

2. Protein quantification

The protein content of each salivary gland pair as determined using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instruction.

3. Salivary gland dissection

Mosquitoes aged between 3 and 7 days after emergence were used. Dissection of various regions of the female salivary glands (Fig. 1) was performed as method described by Jariyapan et al (2010).

4. Protein analysis

SDS-PAGE, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry were used to analyze the salivary gland proteins.

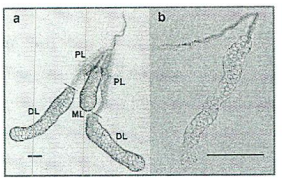

Fig. 1 Representative adult salivary glands of the mosquito, *Anopheles campestris*-like. a: a female salivary gland showing proximal region of the lateral lobe (PL), distal region of the lateral lobe (DL), and median lobe (ML); b: a male salivary gland.

References

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S. Thongsahuan, V. Baimai, Y. Otsuka, et al. *Memo rias do Instituto Oswaldo Cruz.* 104, 558 (2009).
SJ. Kim, W. Choochote, A. Jitpakdi, et al. *Korean J Entomol.* 33, 267 (2003).
W. Choochote, S. Sucharit, W. Abeywickreme. *Southeast Asian J Trop Med Public Health.* 14, 204 (1983).

Acknowledgments

This work was supported by the Thailand Research Fund (Grant No. RMU 5180011 to NJ), the Thailand Research Fund through the Royal Golden Jubilee Ph.D. program (Grant No. PHD/0149/2551) and the Faculty of Medicine Endowment Fund, Chiang Mai University.

Results

1. Determination of *An. campestris*-like salivary gland content

The amount of salivary gland proteins in mosquitoes aged between 3 - 10 days was approximately 1.36 ± 0.04 µg/female (n = 30) and 0.1 ± 0.05 µg/male (n = 30).

2. Analysis of *An. campestris*-like salivary gland proteins by SDS-PAGE (Fig. 2)

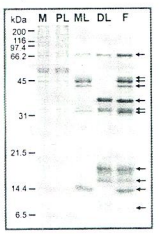

Fig. 2 Female and male salivary gland proteins of *An. campestris*-like. Salivary gland proteins were separated on 15% SDS- polyacrylamide gels and stained with Coomassie blue. M: sixty male salivary glands; PL: fifty female proximal-lateral lobes; ML: two median lobes; DL: two distal-lateral lobes; 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.

3. Analysis of *An. campestris*-like salivary gland proteins by 2D-PAGE (Fig. 3)

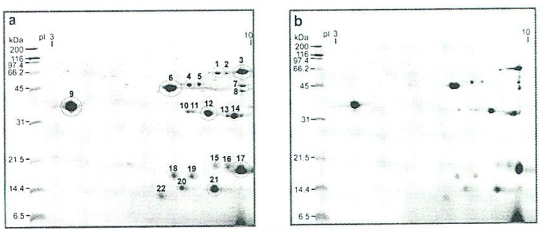

Fig. 3 Coomassie blue-staied 2D gels of female salivary glands of *An. campestris*-like. Numbers indicate major salivary gland proteins. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. a: a representaive of two-dimensional gels of non blood-fed salivary gland proteins from seventy females; b: blood-fed salivary gland proteins from seventy females.

4. NanoLC-MS analysis (Table 1)

Table 1. A list of major protein spots (from Fig. 3a) identified by NanoLC-MS.

Spot numbers	Accession number	Protein score*	Description
1	gi|4582524	62	Apyrase [*An. gambiae*]
2	gi|20857633	51	Putative 5'nucleotidase/apyrase [*An. darlingi*]
3	gi|27372911	56	Salivary apyrase [*An. stephensi*]
4	No match°		
5	No match		
6	No match		
7	No match		
8	No match		
9	gi|190576759	158	Anti-platelet protein [*An. gambiae*]
10	No match		
11	No match		
12	gi|158296846	73	AGAP008279-PA [*An. gambiae* str. PEST]
13	No match		
14	No match		
15	gi|4127333	55	D7r1 protein [*An. gambiae*]
16	gi|4127333	69	D7r1 protein [*An. gambiae*]
17	gi|4127333	56	D7r1 protein [*An. gambiae*]
18	No match		
19	No match		
20	No match		
21	No match		
22	gi|13537666	87	gSG6 protein [*An. gambiae*]

*P < 0.05
°No match to Anopheles sequences

Conclusion

In this work, the female salivary gland protein profiles of *An. campestris*-like were analyzed by 2D-PAGE and the major proteins were identified by LC-MS for the first time. These results provide basic information that would lead to further study on the role of salivary gland proteins of *An. campestris*-like in disease transmission and hematophagy.

เอกสารแนบหมายเลข 6

ผลงานนำเสนอแบบโปสเตอร์ เรื่อง "Two-dimensional gel analysis of salivary gland proteins from female *Anopheles barbirostris* species A2 mosquitoes (Diptera: Culicidae)" ในการประชุมวิชาการ "การประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 10" ที่ โรงแรมฮอลิเดย์ อินน์ รีสอร์ทรีเจนท์ บีช ชะอำ จ.เพชรบุรี วันที่ 14-16 ตุลาคม พ.ศ. 2553

Two-dimensional gel analysis of salivary gland proteins from female *Anopheles barbirostris* species A2 mosquitoes (Diptera: Culicidae)

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Introduction and Objective

Salivary glands of adult female mosquitoes serve a dual function, assisting both blood and sugar meal feeding. Their salivary proteins are also though to play an important role in allergic responses in humans and animals and pathogen transmission. In anopheline mosquito vectors, prior to transmission malaria parasites must reside for some period of time in these organs.

Recently, *A. barbirostris* species A2 has been proven to be a low-potential vector of *P. vivax* in Thailand (Thongsahuan et al, submitted). However, little is known regarding the salivary gland proteins of this mosquito species. Therefore, salivary gland proteins *A. barbirostris* species A2 were analyzed in this study.

Materials and methods

1. Mosquito

A. barbirostris species A2 (Saeung et al, 2008) were successfully maintained for many consecutive generations in an insectary at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand and were used in this study. The methods for rearing mosquitoes described by Choochote et al (1983) and Kim et al (2003) were used.

2. Salivary gland dissection

Salivary glands were dissected from female mosquitoes 0-12, 24-36, and 48-72 hours after emergence and used for age-dependence experiment. Dissection of various regions of the female salivary glands (Fig. 1) was performed as method described by Jariyapan et al (2010).

3. Protein analysis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry were used to analyze the salivary gland proteins.

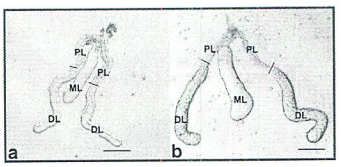

Fig. 1 Representative female salivary glands of *A. barbirostris* species A2. a: a salivary gland from female 1 hr post emergence; b: a salivary gland from female 72 hr post emergence showing proximal region of the lateral lobe (PL), distal region of the lateral lobe (DL), and median lobe (ML). Bar represents 500 µm.

References

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N. Jariyapan, V. Baimai, Y. Poovorawan, et al. *Parasitol Res.* 107, 509 (2010).
S. Thongsahuan, V. Baimai, Y. Otsuka, et al. *Memo rias do Instituto Oswaldo Cruz.* (submitted).
SJ. Kim, W. Choochote, A. Jitpakdi, et al. *Korean J Entomol.* 33, 267 (2003).
W. Choochote, S. Sucharit, W. Abeywickreme. *Southeast Asian J Trop Med Public Health.* 14, 204 (1983).

Acknowledgments

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Results

1. Age-dependence of the salivary gland protein profiles (Fig. 2)

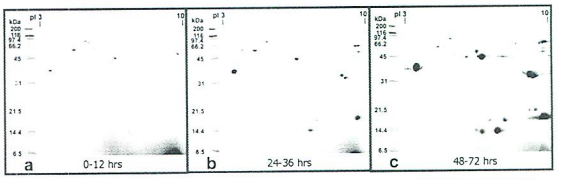

Fig. 2 2D-PAGE analysis of female salivary glands of *A. barbirostris* species A2. Proteins were separated on Immobiline DryStrips 7 cm, pH 3-10. The gels were stained with Coomassie blue. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. **a:** a representaive of 2D gels of 70 gland pairs of female aged 0-12 hrs; **b:** 70 gland pairs of female aged 24-36 hrs; **c:** 70 gland pairs of female aged 48-72 hrs.

2. Protein profiles of different regions of the female salivary glands (Fig. 3)

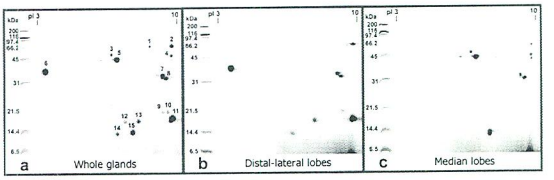

Fig. 3 2D-PAGE analysis of female salivary glands of *A. barbirostris* species A2. Proteins were separated on Immobiline DryStrips 7 cm, pH 3-10. The gels were stained with Coomassie blue. Numbers indicate major salivary gland proteins. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. **a:** a representative of 2D gels of 35 whole female salivary gland pairs; **b:** distal-lateral lobes from 35 females; **c:** median lobes from 35 females.

3. NanoLC-MS analysis (Table 1)

Table 1. A list of major protein spots (from Fig. 3a) identified by NanoLC-MS

Spot numbers	Accession number	Protein score[a]	Description
1	No match[b]		
2	gi|27372911	54	Salivary apyrase (*A. stephensi*)
3	No match		
4	No match		
5	gi|158301626	36	Hypothetical protein (*A. gambiae*)
6	No match		
7	gi|15718081	35	D7 protein (*A. stephensi*)
8	gi|114864717	47	Long form D7 salivary protein (*A. funestus*)
9	No match		
10	No match		
11	gi|4127333	59	D7r1 protein (*A. gambiae*)
12	No match		
13	No match		
14	No match		
15	No match		

[a]$P < 0.05$
[b]No match to *Anopheles* sequences

Conclusion

In this work, the female salivary gland protein profiles of *A. barbirostris* species A2 were analyzed by 2D-PAGE and the major proteins were identified by LC-MS for the first time. These results provide basic information that would lead to further study on the role of salivary gland proteins of *A. barbirostris* species A2 in disease transmission and hematophagy.