รายงานฉบับสมบูรณ์ของโครงการวิจัย

โครงการ

การวิเคราะห์โปรตีนของต่อมน้ำลายในยุงก้นปล่องกลุ่มซับซ้อนชนิด *Anopheles barbirostris* ในประเทศไทย

โดย รองศาสตราจารย์ ดร.นริศรา จริยะพันธุ์

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รายงานวิจัยฉบับสมบูรณ์

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ผู้วิจัย	สังกัด
รศ.ดร.นริศรา จริยะพันธุ์	ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัยซึ่งเป็นผู้สนับสนุนทุนวิจัยในเรื่องนี้ ขอขอบพระคุณ อธิการบดีมหาวิทยาลัยเชียงใหม่, คณบดีคณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ และ หัวหน้าภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ที่อนุญาตและสนับสนุนการวิจัย

ขอขอบพระคุณ ศาสตราจารย์ ดร.เวช ชูโชติ ศาสตราจารย์ยง ภู่วรวรรณ Prof. Dr. Paul Bates ดร.สิทธิรักษ์ รอยตระกูล ที่ช่วยให้คำปรึกษาแนะนำและร่วมทำงานวิจัยอย่างดียิ่ง

รองศาสตราจารย์ ดร.นริศรา จริยะพันธุ์

ABSTRACT

ภาษาไทย:

ยุงก้นปล่องเป็นพาหะสำคัญของมาลาเรียในคน ในการศึกษาครั้งนี้ได้ทำการวิเคราะห์โปรตีนของต่อมน้ำลายของยุงเพศเมีย 5 ชนิด ที่เป็นสมาชิกของยุงกลุ่มซับซ้อน *Anopheles barbirostris* ได้แก่ *Anopheles campestris*-like (Chiang Mai strain), *Anopheles barbirostris* species A1, A2, A3, และ A4 โดยวิธี Sodium dodecyl sulphate polyacrylamide gel eletrophoresis (SDS-PAGE), Two-dimensional gel electrophoresis (2-DE), และ nanoLC-MS การวิเคราะห์ด้วย SDS-PAGE แสดงให้เห็นว่า มีโปรตีนหลักอย่างน้อย 8 แถบและโปรตีนย่อยอีกหลายแถบ ในต่อมน้ำลายของยุงแต่ละชนิด ซึ่งในแต่ละส่วนของต่อมน้ำลายของยุงพบว่า มีโปรตีนหลักแตกต่างกัน รูปแบบของแถบโปรตีนบนเจล SDS-PAGE สามารถแยกชนิดของยุงในกลุ่มซับซ้อนนี้ได้ ความแตกต่างของโปรตีนหลักระหว่างยุงแต่ละชนิดพบในโปรตีนที่มีน้ำหนักระหว่าง 40-48 กิโลดาลตัน 32-37 กิโลดาลตันและ 10-18 กิโลดาลตัน ไม่พบความแตกต่างของรูปแบบของแถบโปรตีนในยุงที่มีรูปแบบโครโมโซมแตกต่างกัน รูปแบบของแถบโปรตีนที่มีหลายรูปแบบพบในยุงชนิด A4 เท่านั้น แถบโปรตีนหลักที่มีน้ำหนักต่ำสุดของยุงแต่ละชนิด (แถบโปรตีนเครื่องหมาย) มีการเคลื่อนที่ที่แตกต่างกันอย่างชัดเจนในบนเจล SDS-polyacrylamide การวิเคราะห์ด้วย nanoLC-MS พบว่า แถบโปรตีนเครื่องหมายนั้นตรงกับโปรตีนที่เกี่ยวข้องกับการกินเลือด gSG6 ของ *Anopheles gambiae* และ *Anopheles freeborni* การวิเคราะห์ด้วย 2-DE แสดงให้เห็นว่า มีโปรตีนหลักอย่างน้อย 14 จุด และโปรตีนย่อยอีกหลายจุดในต่อมน้ำลายของยุงแต่ละชนิด อย่างไรก็ตาม จำนวนโปรตีนหลักที่สามารถบ่งชี้ได้โดยวิธี nanoLC-MS มีจำนวนน้อยกว่าครึ่งหนึ่ง แฟมิลี่ของโปรตีนหลัก 4 แฟมิลี่ที่พบในต่อมน้ำลายของยุงทั้ง 5 ชนิด ได้แก่ apyrase/5’-nucleotidase, anti-platelet (GE-rich/30 kDa), D7/D7-related, และ gSG6 โปรตีนที่พบและ/หรือบ่งชี้ได้ด้วยวิธีการเหล่านี้ สามารถนำไปศึกษาในด้านกลยุทธ์ที่พัฒนาเพื่อควบคุมเชื้อที่ทำให้เกิดโรคและการติดต่อของโรค นอกจากนี้ ข้อมูลแผนที่ 2D ของโปรตีนในต่อมน้ำลายของยุงเพศเมีย อาจจะเป็นประโยชน์ในการสร้างเครื่องมือช่วยในการแยกชนิดของสมาชิกในยุงกลุ่มซับซ้อนนี้

ภาษาอังกฤษ:

Anopheline mosquitoes are the exclusive vectors of human malaria. In this study, the salivary gland proteins of female mosquitoes of the five sibling species in the *Anopheles barbirostris* complex, i.e., *Anopheles campestris*-like (Chiang Mai strain), *Anopheles barbirostris* species A1, A2, A3, and A4 were analyzed by Sodium dodecyl sulphate polyacrylamide gel eletrophoresis (SDS-PAGE), Two-dimensional gel electrophoresis (2-DE), and nanoLC-MS. SDS-PAGE analysis showed that 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 on SDS-PAGE gels distinguished the five sibling species. The variability in major proteins among species was observed in the 40-48 kilodalton (kDa), 32-37 kDa 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*. Two-dimensional gel electrophoresis analysis revealed that at least 14 major and several minor protein spots were detected in the female salivary glands of each species. However, less than half of numbers of the major protein spots of each species were identified by nanoLC-MS. Four protein families were found in the salivary glands of the five sibling species including apyrase/5’-nucleotidase, anti-platelet (GE-rich/30 kDa), D7/D7-related, and gSG6. Proteins detected and/or identified by these approaches could be tested in strategies developed to control pathogen and disease transmission. Moreover, the information of the 2D maps of the female salivary gland proteins might be useful for construction of an additional tool to distinguish species members in the complex.

EXECUTIVE SUMMARY

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. 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*. Recently, *An. barbirostris* complex, i.e., *An. campestris*-like (Chiang Mai strain), *An. barbirostris* species A1, A2, A3, and A4 of Thai populations were discovered and tested for susceptibility to indigenous strains of *Plasmodium vivax*. The results showed that *An. campestris*-like (Form B and E, Chiang Mai strain) was a high potential vector whereas *An. barbirostris* species A1, A2, and A3 were low potential vectors. For *An. barbirostris* species A4, it was a refractory vector for *P. vivax*. As all are morphologically indistinguishable, incorrect identification of individual members in the complex may result in failure to distinguish between a vector and non-vector species, and lead to the complication and/or unsuccessful formation of vector control strategies. Although the ITS2 sequence of species A4 (1,676 bp) can be used to distinguish species A1 (1,861 bp), A2 (1,717 bp), and A3 (1,070 bp), it is slightly different from *An. campestris*-like (1,651 bp). Thus, it is difficult to differentiate the size of the ITS2-PCR products between species A4 and *An. campestris*-like on a 0.8% agarose gel. In sandflies, electrophoretic profiles of salivary proteins are able to distinguish phlebotomine species. Also, electrophoresis of salivary heme proteins could be used to identify morphologically similar *Rhodnius* species. In addition, salivary gland proteins and/or genes are proposed to be a useful tool for further analysis of the *Anopheles darlingi* taxonomic status. Therefore, in this study, the salivary glands of female mosquitoes of the five sibling species in the *An. barbirostris* complex were analyzed by SDS-PAGE, 2-DE, and nanoLC-MS.

SDS-PAGE analysis showed that 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 kilodalton (kDa), 32-37 kDa 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 *An. gambiae* and *An. freeborni*. Two-dimensional gel electrophoresis analysis revealed that at least 14 major and several minor protein spots were detected in the female salivary glands of each species. However, less than half of numbers of the major protein spots of each species were identified by nanoLC-MS. Four protein families were commonly found in the salivary glands of the five sibling species including apyrase/5'-nucleotidase, anti-platelet (GE-rich/30 kDa), D7/D7-related, and gSG6.

Detail analyses on the expression of salivary gland proteins in mosquitoes aged varying from 0 to 60 hours post emergence and the differential distribution of salivary components within the glands of female mosquitoes were performed in *An. barbirostris* species A2. 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 kilodaltons and isoelectric points ranging from 3.9 to 10. The proteome profiles of *An. barbirostris* species A2 female salivary glands were affected by ageing. The typical electrophoretic pattern of the female salivary glands was reached in 48 hours 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 proteins start to accumulate from emergence and gradually increase becoming predominant within 48 hours. 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.

In addition, *An. campestris*-like salivary gland proteins were determined and analyzed. 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. 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.

Proteins detected and/or identified by these approaches could be tested in strategies developed to control pathogen and disease transmission. Moreover, the information of the 2D maps of the female salivary glands might be useful for construction of an additional tool to distinguish species members in the complex. Furthermore, because of differences in their roles in the transmission of malaria and filariasis, description of the salivary proteomes and transcriptomes of the *An. barbirostris* complex is required. Comparative analysis of the proteomes/transcriptomes of the sibling species may supply better tools for determination of phylogeny of closely related species, population structure and speciation processes, and ultimately, identification of genes related to vectorial capacity and host preference.

เนื้อหางานวิจัย

CHAPTER 1

INTRODUCTION

Anopheline mosquitoes are the exclusive vectors of human malaria. Salivary glands are of interest in the 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 produce a wide array of secreted compounds that are delivered with the saliva and help blood-feeding by affecting the host hemostatic response. In addition, the saliva is the vehicle that carries pathogens and may also enhance or facilitate infectivity during the blood meal. Analyses of salivary gland proteins (sialomes) and/or genes (transcriptomes) of anopheline mosquitoes were performed in *Anopheles gambiae*, *Anopheles stephensi*, *Anopheles darlingi*, and *Anopheles funestus*. The sequences of the mosquito sialotranscriptomes were grouped in protein/gene families. Recently, some of secreted protein(s)/gene(s) are proposed to use as a molecular marker for assessing phylogeny among closely related species in *An. darlingi* complex, an important human malaria vector in Brazil, as has been demonstrated with triatomine bugs using the salivary heme proteins.

In Thailand, anthropophilic *Anopheles barbirostris*/*campestris* were recently incriminated as potentially natural vectors of *Plasmodium vivax* in the Aranyaprathet district of Sa Keao province. They were also considered as possible vectors playing an important role in increasing cases of *P. vivax* infection in this country. As morphologically indistinguishable, adult females of *Anopheles barbirostris* and *Anopheles campestris* lead to marked errors in identification between these two species in the study of malaria epidemiology and control. Saeung et al. (2007) investigated the anthropophilic *An. barbirostris/campestris* group strain from Chiang Mai province and revealed that besides the 4 forms of metaphase karyotypes of *An. barbirostris* [Form A: X_2, X_3, Y_1, B: X_1, X_2, X_3, Y_2, C: X_2, X_3, Y_3 and D: X_2, Y_4 (found only in Indonesia)] and the typical *An. campestris* (X, Y) strain from Ayutthaya province, there is a new karyotypic form (Form E: X_2,Y_5). The summation of branches of seta 2-VI of pupal skins 22.25 (18-30) is in the range of *An. campestris*, whereas its characteristics of metaphase karyotypes, particularly the chromosome Y_5, is markedly different from *An. barbirostris* Form A, B, C, D, and the typical *An. campestris* strain. Thus, it is tentatively designated *An. campestris*-like Form E. The hybridization of sympatric *An. campestris*-like Form E with *An. barbirostris* Form B demonstrated strong reproductive isolations. In addition, the crossing experiments between two allopatric strains of *An. barbirostris* Form A (Chiang Mai and Phetchaburi) also indicated extensive reproductive isolations (Choochote et al. unpublished data). Choochote et al (personal communication) have investigated karyotypic variations of the *An. barbirostris* complex in the systematic direction and found that the *An. campestris*-like Form E is susceptible in a high level to *P. vivax* in laboratory but the *An. barbirostris* Form A is not a malaria vector. This result corresponds to the report of Apiwathnasorn et al (2002).

Despite their importance as malaria vectors, no protein/nucleotide sequence of the *An. barbirostris* complex salivary gland is available in the NCBI database. The comparative analysis of the proteomes and/or transcriptomes of the mosquito members in this complex that having distinct primary host [human (vector) or other animals (non-vector)], may supply better tools for determination of phylogeny of closely related species, population structure and speciation processes, and ultimately identify genes related to vectorial capacity and host preference. All of this information is likely to be useful for the improvement of existing and development of novel transmission-reduction malaria control strategies. Therefore, in this study, electrophoretic protein profiles of female salivary glands of each form and sibling species in the *An. barbirostris* complex from different regions in Thailand were analyzed using Sodium dodecyl sulphate polyacrylamide gel eletrophoresis (SDS-PAGE), Two-dimensional gel electrophoresis (2-DE), and nanoLC-MS.

CHAPTER 2

LEITERATURE REVIEWS

Malaria remains the most prevalent and devastating insect-borne parasitic disease in humans, with 255 million cases reported worldwide yearly. However, the number of deaths due to malaria is estimated to have decreased from 985,000 in 2000 to 781,000 in 2009 (WHO 2010). In Thailand, four species of human malaria parasites are found; the most common being *P. vivax* (50.74%) and *Plasmodium falciparum* (48.61%), while *Plasmodium malariae* (0.20%) and *Plasmodium ovale* (one case reported from Chiang Mai Province in 1996) are rare, and 0.45% are mixed infections. The disease is generally limited to rural communities living in and near forested regions, mountains and foothills, particularly those people residing in newly opened land settlements of semi-forested areas, where they earn their living by growing agricultural crops. Regions near and along the borders with neighboring countries, i.e., Cambodia, Laos, Myanmar and Malaysia, are also affected.

Anopheline mosquitoes are the exclusive vectors of human malaria. Salivary glands are of interest in the 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 produce a wide array of secreted compounds that are delivered with the saliva and help blood-feeding by affecting the host hemostatic response. In addition, the saliva is the vehicle that carries pathogens and may also enhance or facilitate infectivity during the blood meal (Ribeiro and Francischetti 2003). Analyses of salivary gland proteins (sialomes) and/or genes (transcriptomes) of anopheline mosquitoes were performed in *An. gambiae* (Arca et al. 1999a, b, 2005; Dana et al. 2005; Kalume et al. 2005a, b; Francischetti et al. 2002; Lanfrancotti et al. 2002), *An. stephensi* (Valenzuela et al. 2003), *An. darlingi* (Calvo et al. 2004), *An. funestus* (Calvo et al. 2007) and *An. dirus* B [= *cracens* (Sallum et al. 2005a)] (Jariyapan et al. unpublished data). Calvo et al (2004) compared *An. darlingi* (subgenus *Nyssorhynchus*) and *An. gambiae* (subgenus *Cellia*) salivary gland genes belonging to the secreted and housekeeping categories. They concluded that the salivary gland genes encoding secreted products are rapidly evolving in comparison with the housekeeping genes of these species. Valenzuela et al (2003) found similar results when the salivary gland transcriptomes of *An. stephensi* and *An. gambiae* were compared. These two species belong to the same subgenus (*Cellia*) and when compared showed 93% of identity for gene products of the housekeeping group whereas the salivary proteins are only 62% identical. These results support the idea that secreted genes may be good markers for assessing phylogeny among closely related species, as has been demonstrated with triatomine bugs using the salivary hemeproteins (Soares et al. 1998, 2000). In *An. darlingi,* previous studies based on behavioural (patterns of biting activity), morphological (body size and polytene chromosome patterns) and molecular (allozymes and ITS2 sequences) differences among geographically distinct populations have indicated the possibility that *An. darlingi* is a complex of closely related species (Lounibos and Conn 2000). Salivary gland proteins and/or genes are proposed to be a useful tool for further analysis of the *An. darlingi* taxonomic status (Calvo et al. 2004).

In Thailand, there are at least 18 anopheline species playing an important role as primary, secondary and suspected vectors of malaria transmission. The primary vectors are *Anopheles dirus* complex [*Anopheles dirus s.s.* (species A)*, Anopheles baimaii* (species D)], *Anopheles minimus* complex [*An. minimus s.l.* (species A)] and *Anopheles maculatus* complex [*Anopheles maculatus s.s.* (species B)], while *Anopheles aconitus* and *Anopheles sundaicus* complex [species A = *epiroticus* (Linton et al. 2005)] are considered as secondary vectors (Gould et al. 1967; Scanlon et al. 1968; Harrison 1980; Rosenberg et al. 1990; Rattanarithikul et al. 1996; Subbarao 1998; Sallum et al. 2005a, b). Subsequently, *Anopheles pseudowillmori*, a member species of the *maculatus* complex, has been incriminated as a secondary vector (Green et al. 1991). Recently, *An. campestris* (identification was based only on the summation of seta 2-VI branches of pupal skins) was incriminated as a potentially natural vector of *P. vivax* in Pa Rai subdistrict of Aranyaprathet district, Sa Kaeo province (Apiwathnasorn et al. 2002). The remaining 11 species, i.e., *Anopheles annularis*, *Anopheles karwari*, *Anopheles kochi*, *Anopheles nigerrimus*, *Anopheles nivipes*, *Anopheles peditaeniatus*, *Anopheles philippinensis*, *Anopheles sawadwongporni*, *Anopheles sinensis*, *Anopheles tessellates*, and *Anopheles vagus* are suspected vectors, since they were found positive by an ELISA method for oocysts in the midgut and/or circumsporozoite antigens (Baker et al. 1987; Harbach et al. 1987; Gingrich et al. 1990; Frances et al. 1996; Rattanarithikul et al. 1996).

The anthropophilic *An. barbirostris*/*campestris* group has been reported firstly as a probable vector of malaria in Pa Rai subdistrict, Aranyaprathet district, Sa Kaeo province (Limrat et al. 2001). The increase in population, high biting density, anthropophilicity, high susceptibility to *P. vivax* and detection of circumsporozoite protein (Pv 247) (Somboon et al. 1994; Frances et al. 1996; Limrat et al. 2001; Apiwathnasorn et al. 2002), have caused this species group to be considered as a possible, important vector corresponding to the increase of *P. vivax* prevalence in Thailand (Sattabongkot et al. 2004). Based on the identification of pupal skins, Apiwathnasorn et al (2002) subsequently incriminated *An. campestris* as a potentially natural vector of *P. vivax* in this locality, although investigations into the summation of branches of seta 2-VI of 500 pupal skins, derived from 50 iso-female lines, revealed 94.4% of 17-58 branches (*An. campestris*), 14.8% of 17-18 branches (overlapping), and 5.6% of < 18 branches *(An. barbirostris*). The crucial question as to whether the 14.8% overlapping range is *An. campestris*, *An. campestris*-like or *An. barbirostris* is still ambiguous. Exact species identification by using metaphase karyotypes (Baimai et al. 1995), which have so far been proven as the only reliably diagnostic tools, should be intensively carried out prior to drawing the final conclusions.

Little is known about the *An. barbirostris*/*campestris* group from the population genetic point of view, or its exact role as a vector of malaria in many localities of Thailand. Reproductive isolation between two strains of *An. barbirostris* from Chon Buri and Chumporn province were first demonstrated by Choochote et al (1983). The results indicated that these two strains exhibit a possible presence of a species complex. Subsequently, three karyotypic forms of *An. barbirostris* (Form A: X_2, X_3, Y_1; Form B: X_1, X_2, X_3, Y_2; and Form C: X_2, X_3, Y_3) have been reported throughout Thailand, whereas Form D (X_2, Y_4) has been reported from Java, Indonesia by Baimai et al (1995). Recently, an additional, new karyotypic form of *An. campestris*-like [based on the summation of seta 2-VI branches of pupal skins 22.25 (18-30)], designated Form E (X_2, Y_5), was obtained from Chiang Mai province, northern Thailand (Saeung et al. 2007). Its metaphase karyotype, particularly the Y-chromosome is markedly distinct from the four forms of *An. barbirostris* and *An. campestris*, i.e., Y_1 is subtelocentric, Y_2 is large submetacentric, Y_3 is large submetacentric or metacentric, and Y_4 is medium metacentric in *An. barbirostris* Form A, B, C, and D, respectively; Y_5 is small metacentric in *An. campestris*-like Form E; and Y is telocentric in typical *An. campestris*. In addition, investigations of post-mating barriers by hybridization between sympatric *An. campestris*-like Form E and *An. barbirostris* Form B revealed strong genetical incompatibility, providing low embryonation and hatchability rates, retained stage and inviability of hatched larvae. Another interesting point is that the crossing between two allopatric *An. barbirostris* Form A strains from Chiang Mai and Phetchaburi province demonstrated extensive reproductive isolations, providing low viability of F_1-hybrid larvae with asynaptic salivary gland polytene chromosomes, sex distortion of adults, abnormal development of ovarian follicles of F_1-hybrid females, and atrophy of the accessory glands and testes of F_1-hybrid males. Choochote et al (personal communication) have investigated karyotypic variations of the *An. barbirostris* complex in the systematic direction and found that the *An. campestris*-like Form E is susceptible in a high level to *P. vivax* in laboratory but the *An. barbirostris* Form A is not a malaria vector. This result corresponds to the report of Apiwathnasorn et al (2002).

Despite their importance as malaria vectors, no protein/nucleotide sequence of the *An. barbirostris* complex salivary glands is available in the NCBI database. The comparative analysis of the proteomes and/or transcriptomes of the mosquito members in this complex that having distinct primary host [human (vector) or other animals (non-vector)], may supply better tools for determination of phylogeny of closely related species, population structure and speciation processes, and ultimately identify genes related to vectorial capacity and host preference. All of this information is likely to be useful for the improvement of existing and development of novel transmission-reduction malaria control strategies.

CHAPTER 3

MATERIALS AND METHODS

3.1 Mosquito collection and isoline colonization

Wild-caught, fully engorged females of the *An. barbirostris* complex were collected during June 2008 to June 2009 in 8 provinces in Thailand (Fig. 1 and Table 1), where different populations of the *An. 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).

3.2 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).

3.3 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 amplified by polymerase chain reaction (PCR) using the following primers: ITS2A, 5'-TGTGAACTGCAGGACACAT-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).

3.4 Salivary gland dissection

The mosquitoes were cold anaesthetized 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 (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. 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 a new the 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 $-80^{\circ}C$ until use.

3.5 Sodium dodecyl sulphate polyacrylamide gel eletrophoresis (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), 100mM 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 15 % SDS polyacrylamide gels. Protein molecular weight markers (Bio-Rad Laboratories; Hercules, CA) were applied in each gel.

3.6 Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis 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 ($\approx$ 90 µg of total proteins) were used. For the male sample, 200 pairs of salivary glands ($\approx$ 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.

3.7 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.

3.8 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).

3.9 In-gel digestion

Protein bands or spots of interest were excised from the SDS-polyacrylamide gels or 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 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 (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. 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.

3.10 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).

CHAPTER 4

RESULTS AND DISCUSSION

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

RESULTS

Mosquito collection and identification of mosquito forms and species

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

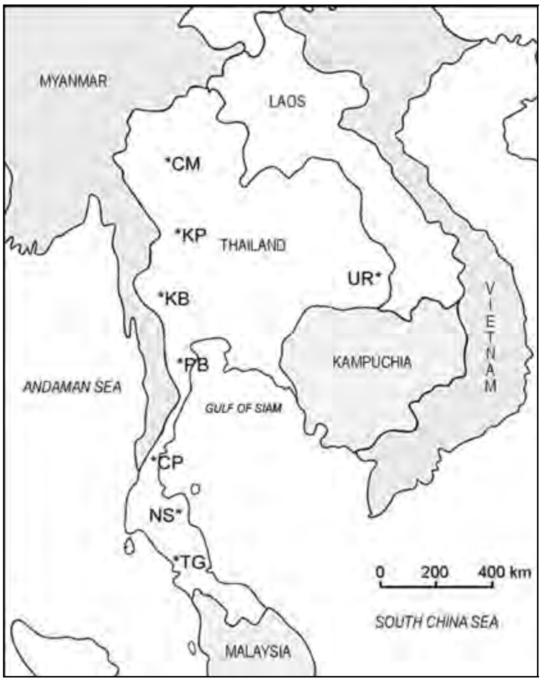


Fig. 1 Collection sites (*) for the *An. barbirostris/campestris* group in Thailand. CM: Chiang Mai; KP: Kamphaeng Phet; KB: Kanchanaburi; UR: Ubon Ratchathani; PB: Phetchaburi; CP: Chumphon; NS: Nakhon Si Thammarat; TG: Trang.

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

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

[a] h – human bait; a – animal bait

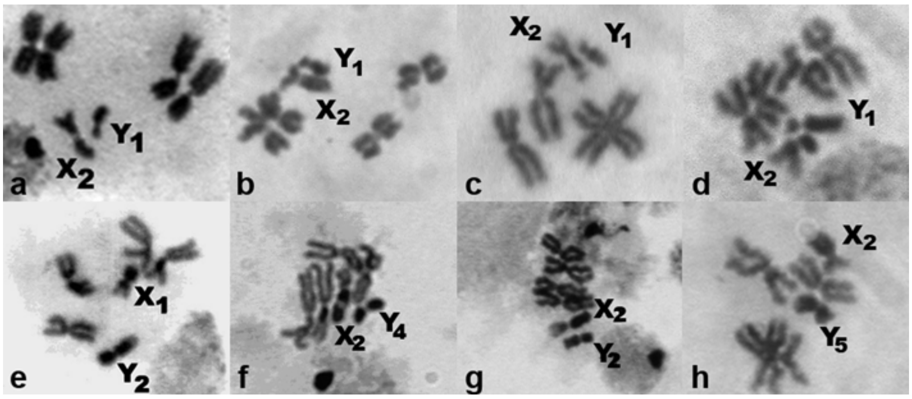


Fig. 2 Metaphase karyotypes of *An. barbirostris* complex. **(a)** species A1, Form A ($X_{2,}$ Y_1: Chiang Mai); **(b)** species A2, Form A (X_2, Y_1: Phetchaburi); **(c)** species A3, Form A (X_2, Y_1: Kanchaburi); **(d)** species A4, Form A ($X_{2,}$ Y_1: Chiang Mai); **(e)** species A1, Form B ($X_{1,}$ Y_2: Chumphon); **(f)** species A1, Form D ($X_{2,}$ Y_4: Nakhon Si Thammarat); **(g)** *An. campestris*-like, Form B ($X_{2,}$ Y_2: Kamphaeng Phet); **(h)** *An. campestris*-like, Form E ($X_{2,}$ Y_5: Chiang Mai).

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

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

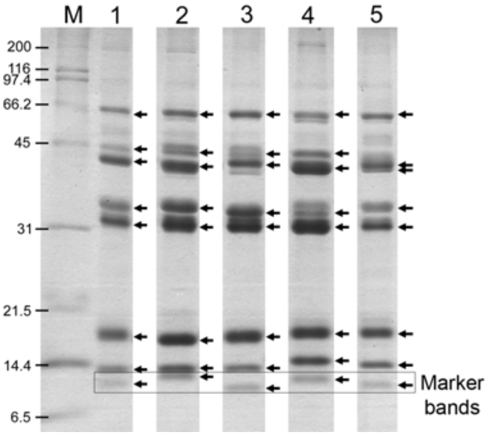


Fig. 3 Electrophoretic profiles of salivary glands obtained from female *An. barbirostris* complex. Proteins of one salivary gland pair were separated on 15% SDS-polyacrylamide gels and stained with Coomassie brilliant blue. Lane 1, *An. barbirostris* species A1; lane 2, species A2; lane 3, species A3;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

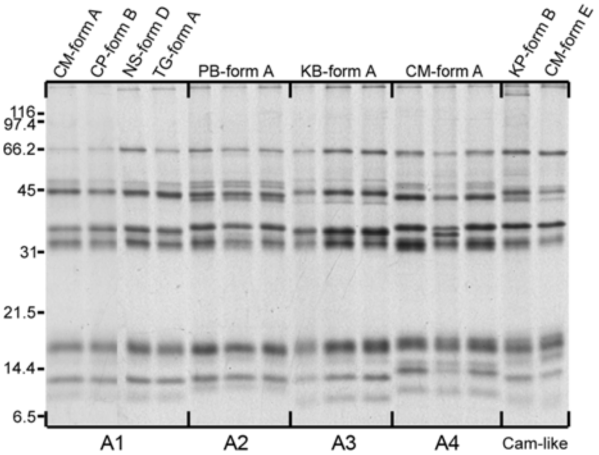


Fig. 4 Electrophoretic protein profiles of salivary glands obtained from female *An. barbirostris* species A1, A2, A3, A4, and *An. campestris*-like. Proteins of one pair of salivary gland were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Mosquitoes from CM: Chiang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

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

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

[a] No match to *Anopheles* sequences

[b] $P < 0.05$

Investigation of the proteins in each salivary gland lobes

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

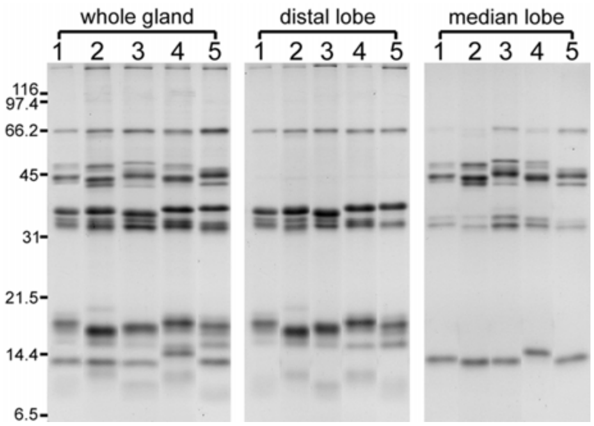


Fig. 5 Electrophoretic protein profiles of salivary glands obtained from female *An. barbirostris* species A1, A2, A3, A4, and *An. campestris*-like. Proteins of one pair of whole salivary gland or distal lobe or median lobe were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie brilliant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

DISCUSSION

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

In this study, we collected mosquitoes in the same and other localities for the *An. barbirostris* complex in eight provinces in Thailand to confirm the distribution of each species. The result correlates with previous collection data (Saeung et al. 2007, 2008; Suwannamit et al. 2009; Thongsahuan et al. 2009). *An. barbirostris* species A1 and A2 were widely distributed in sympatry in low land areas in the north, northeast, south, and central Thailand, whereas species A3 and A4 were confined to Kanchanaburi and Chiang Mai, respectively. *An. campestris*-like was found at a lower altitude in rice paddy fields in San Sai District, Chiang Mai. Species A4 was detected in sympatry with species A1 at high altitude near forested foot hills of Maetang District, Chiang Mai, about 30 km from San Sai District.

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

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

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

Polymorphism of the protein profiles mosquito within species was only noted at 36 kDa protein of *An. barbirostris* species A4. This suggests that salivary proteins of this species might be more complex than those of other sibling species. Transcriptome and proteome analysis of this species and investigation of the degree of intra-specific salivary gene polymorphism in natural mosquito populations should be performed to clarify this issue.

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

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

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

RESULTS

Age dependence of salivary gland protein profiles

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

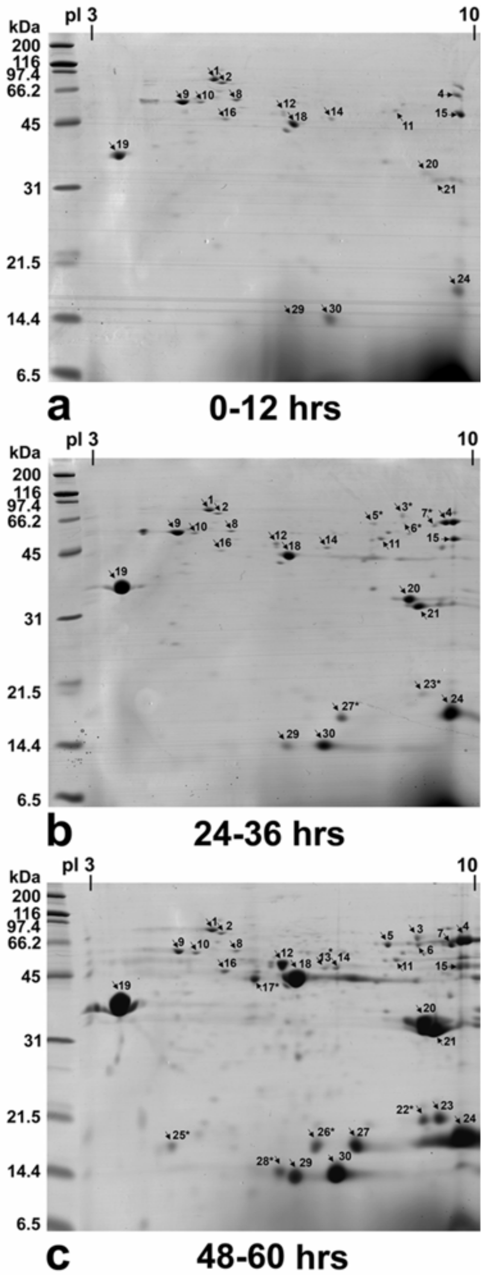


Fig. 1 Two-dimensional gel analysis of female salivary gland proteins of *An. barbirostris* species A2 mosquitoes according to age. Proteins were separated on Immobiline DryStrips 7 cm, pI 3-10. Separation in the second dimension was performed using 15% SDS-PAGE. The gels were stained with Coomassie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

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

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

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

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

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

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

[c] NSH: Not significant hit

[d] $P < 0.05$ or MASCOT score ≥ 30

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

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

[g] Low amount of expression

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

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

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

[b] MW: observed molecular mass

[c] pI: observed isoeletric point

[d] 0-12 hours post emergence

[e] 24-36 hours post emergence

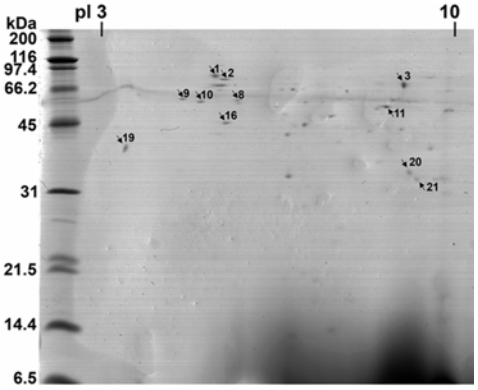
[f] 48-60 hours post emergence

[g] ANV: average normalization volume of protein spot

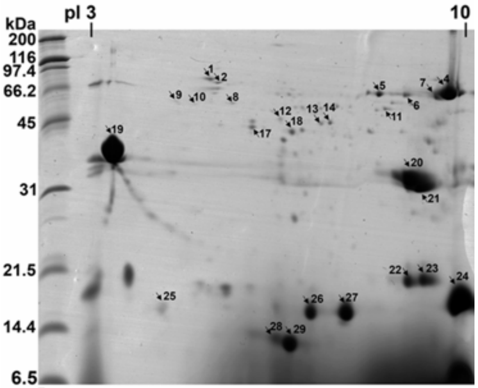
[h] Index: relative volume expressed on the hour when the relatively average normalization volume of protein was expressed at the first hour

Protein profiles of different regions of the female salivary glands

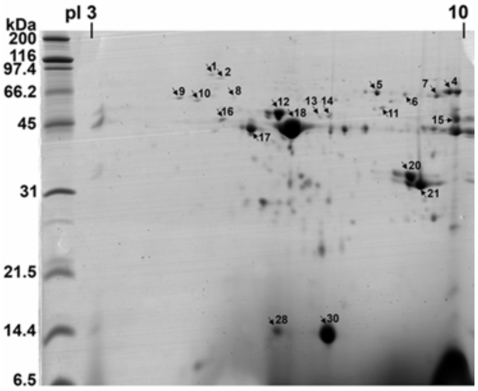
The results of the 2-DE gels of total proteins in the proximal-lateral lobes, distal-lateral lobes and median lobes of the female salivary glands of *An. barbirostris* species A2 are shown in Figure 2. The different morphological regions of the female salivary glands displayed distinct electrophoretic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



a Proximal-lateral lobes



b Distal-lateral lobes



c Median lobes

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

DISCUSSION

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

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

Apyrases are nucleoside triphosphate-diphosphohydrolases present in a variety of organisms. In *Ae. aegypti*, the salivary apyrase was identified as a member of the 5' nucleotidase family (Champagne et al. 1995). Recently, apyrase of *Ae. albopictus* was cloned and characterized (Dong et al. 2012). It is an enzyme that helps the acquisition of blood meals by the degradation of adenosine diphosphate (ADP), a mediator of platelet aggregation and inflammation (Ribeiro and Francischetti 2003) and prevents neutrophil activation (Sun et al. 2006). Smartt et al (1995) showed that apyrase protein levels peak in the salivary glands about four days after adult emergence and remain high after a blood meal. The biochemical analyses of the salivary glands of female *An. darlingi* (Marinotti et al. 1996) and *An. dirus* B (Jariyapan et al. 2007) revealed the presence of apyrase activity in the distal region of the lateral lobes (~66%), median lobe (~23%) and proximal region of the lateral lobes (~11%). In this study, *An. barbirostris* species A2 apyrase was found only in distal-lateral and medial lobes. No apyrase was detected in the proximal-lateral lobe by 2-DE approach. Characterization of apyrase activity in the *An. barbirostris* species A2 would help to clarify this issue.

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

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

Another protein involved in blood feeding is gSG6. It was detected in the distal-lateral lobes of the salivary gland of *An. barbirostris* species A2. This result correlates with a study in *An. gambiae* that gSG6 was expressed only in distal-lateral lobes (Lombardo et al. 2009). The gSG6 protein was first identified in the female *An. gambiae* mosquito (Lanfrancotti et al. 2002). It was conserved in five species members of the *An. gambiae* complex, i.e., *An. gambiae*, *Anopheles melas*, *Anopheles bwambae*, *Anopheles quadriannulatus* A, and *Anopheles arabiensis* (Lombardo et al. 2009). The protein was secreted with the saliva while the female mosquito probes for feeding. Injection of *gSG6* dsRNA into adult *An. gambiae* females resulted in decreased gSG6 protein levels, increased probing time and reduced blood feeding ability (Lombardo et al. 2009). Lombardo et al. (2009) concluded that gSG6 plays some essential blood feeding role in female mosquitoes. Recently, the gSG6-P1 peptide from gSG6 protein of *An. gambiae* salivary glands was designed as a specific salivary sequence of malaria vector species. It was shown that the quantification of human antibody (Ab) responses to *Anopheles* salivary proteins in general and especially to the gSG6-P1 peptide was a pertinent biomarker of human exposure to *Anopheles* (Poinsignon et al. 2008; Drame et al. 2010).

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

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

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

Five and two protein spots significantly matched with sequences of hypothetical proteins of *An. gambiae* and *C. quinquefasciatus*, respectively. Two of them, AGAP007393-PB [*An. gambiae* str. PEST] (SN8) and AGAP012407-PA [*An. gambiae* str. PEST] (SN9), decreased in volume after emergence. However, the function of the proteins is still unknown as their sequences have no obvious protein domain or motif that can provide some clues regarding their function. Thirteen protein spots were not significantly matched with protein sequences in the database. These proteins should be identified with other proteomic approaches, for examples, 1-DE followed by LC MS/MS, in-solution followed by LC MS/MS, and iTRAQ labelling. These approaches may help to identify more proteins in the salivary glands as the previous studies in *An. gambiae* (Kalume et al. 2005a; Choumet et al. 2007).

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

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

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

RESULTS

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

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

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

Twelve major protein bands of the *An. campestris*-like female salivary glands are shown in Figure 1. The male protein profile differed from the whole female profile (compare lane M with lane F) but appeared similar to the proximal-lateral region profile (lane PL). The different morphological regions of the female salivary glands also displayed distinct protein profiles. Female specific protein bands 1, 5, 6, 7, 8, 9, 10 and 12 appeared in the distal region (lane DL) whereas the protein bands 1, 2, 3, 4, 6, 7 and 11 were in the medial lobe (lane ML). For more detail analysis on the expression patterns of proteins in the male and different lobes of female salivary glands 2-DE and nanoLC-MS were performed. Each sample was subjected to triplicate runs, and the results were highly reproducible. Two-dimensional gel electrophoresis experiments provided evidence of many proteins in the female mosquito salivary glands, approximately 85 well-resolved spots (Fig. 2a). The gel (Fig. 2a) was considered the standard reference gel. The molecular mass of these spots varied from 10-72 kDa, with pI ranging of 3.9-10. Twenty major protein spots were detected in the female mosquitoes and identified by nanoLC-MS. Spot numbers in Table 1 correspond to the salivary gland proteins shown in Figure 2a. From the 20 major protein spots, 15 spots are positive for glycoprotein staining (Fig. 2b and Table 1). Expression patterns of the protein spots in male and different lobes of female salivary glands are shown in Figure 3 and Table 1. Ten major protein spots (SN1, 2, 3, 4, 6, 8, 11, 12, 15, and 19) were similarly found with weak intensity in the male salivary glands (Fig. 3a) and the proximal-lateral lobes of female glands (Fig. 3b). Five major protein spots (SN13, 14, 16, 17, and 20) were detected only in the distal-lateral lobes (Fig. 3c) whereas three major spots (SN5, 7, and 18) were specific in the medial lobe (Fig. 3d).

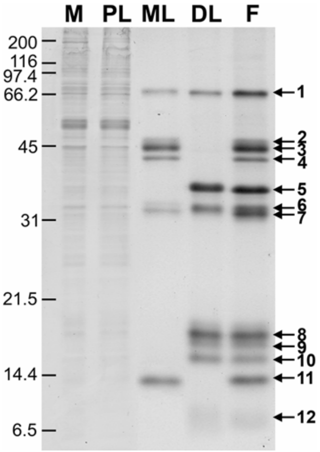


Fig. 1 Female and male salivary gland protein profiles of *An. campestris*-like. The salivary gland proteins were separated on 15% SDS-polyacrylamide gels and stained with CBB. M: sixty male salivary glands; PL: fifty female proximal-lateral lobes; ML: two median lobes; DL: two distal-lateral lobes;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

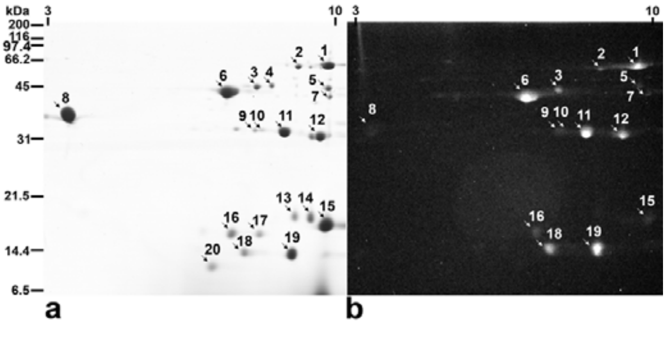
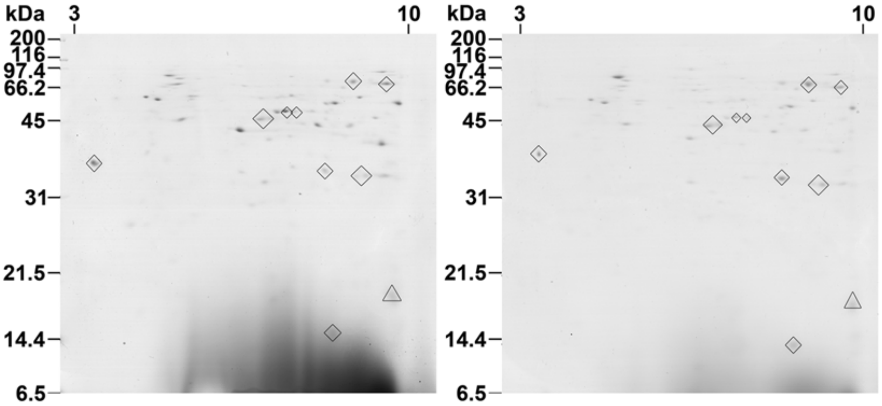
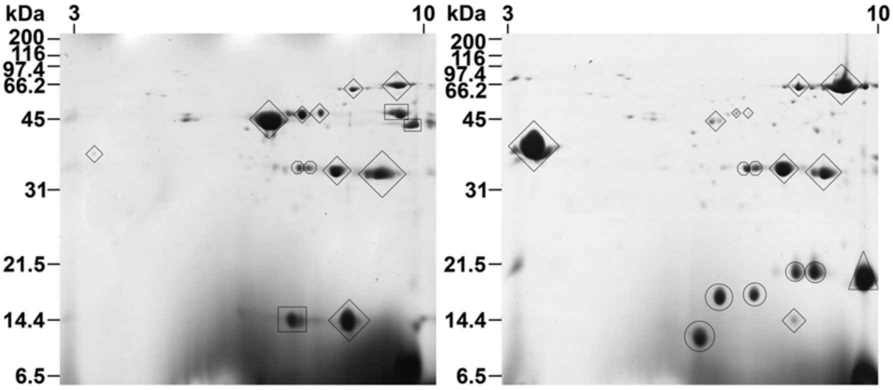


Fig. 2 Two-dimensional gel analysis of female salivary gland proteins of *An. campestris*-like. Proteins were separated on Immobiline DryStrips 7 cm, pI 3-10. Separation in the second dimension was performed using 15% SDS-PAGE. Molecular mass markers are indicated on the left in kDa. Isoelectric points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



a **Male whole glands** **b** **Proximal-lateral lobes**



c **Median lobes** **d** **Distal-lateral lobes**

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

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

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

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

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

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

[c] NSH = not significant hit

[d] $P < 0.05$ or MASCOT score ≥ 30

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

[f] Low amount of expression

[g] Low intensity

DISCUSSION

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

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

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

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

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

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

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

Most 5' nucleotidases are typically extracellular proteins bound to the membrane by glycosylphosphatidylinositol (GPI) anchors attached to their carboxyterminal domain. However, 5' nucleotidase/apyrases in several insects including *Ae. aegypti*, *Ae. albopictus*, *Culex pipiens* *quinquefasciatus*, *L. longipalpis*, and *Glossina morsitans morsitans* lack the GPI anchor attachment domain, either through mutation or truncation, thus inferring that these proteins are secreted (Champagne et al. 1995; Charlab et al, 1999; Ribeiro et al. 2004; Ribeiro et al. 2007;Alves-Silva et al. 2010; Dong et al. 2012). An acidic glycoprotein of 35 kDa (GP35 ANOAL) from female salivary glands of *An. albimanus* contains several potential post-translational modifications predicted in its amino acids sequence. Among them, two potential N-glycosylation and nine potential O-glycosylation sites have been identified (Cazares-Raga et al. 2007). Putative N-glycosylation and O-glycosylation sites occur in all identified members of the 30kDa allergen family (Xu et al. 1998; Ribeiro et al. 2003; Valenzuela et al. 2003; Calvo et al. 2004; Jariyapan et al. 2006; Cazares-Raga et al. 2007; Yoshida et al. 2008). Proteins that contain N-glycosylation sites may play important roles in the induction of allergic responses (Wal 2001; Malandain 2005). For D7 family proteins, only D7 long forms of *An. gambiae*, *Anopheles stephensi*, *Anopheles arabiensis*, *Anopheles funestus*, and *Anopheles darlingi* contain glycosylation sites (Francischetti et al. 2002; Suwan et al. 2002; Valenzuela et al. 2002; Calvo et al. 2007; Calvo et al 2009). Glycoproteins in the salivary glands of mosquitoes should be studied for their structure and specific biological functions in receptor binding, cell signaling, and immune recognition that may be involved in pathogen transmission.

Three major protein spots of *An. campestris*-like salivary glands related to housekeeping proteins from other arthropod species included serine/threonine-protein kinase rio3 [*Culex quinquefasciatus*], sil1, putative [*Ixodes scapularis*], and cyclophilin A, putative [*I. scapularis*]. SN6 Protein that matched the serine/threonine-protein kinase rio3 of *Culex quinquefasciatus* (Arensburger et al. 2010) was a protein expressed abundantly in the medial lobe of female salivary glands but found with a small amount in the both region of the lateral lobes and the male salivary glands. The RIO family of atypical serine protein kinases has been first characterized in *Saccharomyces cerevisiae* (Angermayr and Bandlow 1997). It consists of enzymes that contain a unique domainwith a characteristic kinase sequence motif and usually some additional domains. At least two RIO proteins, Rio1 and Rio2, are present in organisms varying from Archaea to humans, with a third Rio3 subfamily present only in multicellular eukaryotes (Manning et al. 2002). Human Rio3 is characterized at the DNA level only, in two splice variants, and is identified as a protein up-regulated in the core of malignant melanomas (Roesch et al. 2003). To date, no report of the purification of mosquito kinase Rio3 is available. SN 10 matched sil1, putative [*Ixodes scapularis*]. It was predominantly expressed in both the medial and distal-lateral lobes. SIL1 in *Homo sapiens* is a resident endoplasmic reticulum (ER), N-linked glycoprotein with an N-terminal ER targeting sequence, 2 putative N-glycosylation sites, and a C-terminal ER retention signal. This protein is required for protein translocation and folding in the endoplasmic reticulum (ER). It functions as a nucleotide exchange factor for the heat-shock protein 70 (HSP70) chaperone HSPA5 (Anttonen et al. 2005). SN 14 matched cyclophilin A, putative [*I. scapularis*]. It was expressed specifically in the distal-lateral lobe. Cyclophilin A also known as peptidylprolyl isomerase A is the most abundant member of the CyP subfamily of immunophilins and has a variety of intracellular functions, including intracellular signaling, protein trafficking, and the regulation of other proteins activity. In humans, cyclophilin A has been studied as a multifunctional protein that is up regulated in a variety of inflammatory conditions, such as rheumatoid arthritis, autoimmune disease, and cancer. Besides its intracellular functions, CyPA is a secreted molecule that has a physiological and pathological role in cardiovascular diseases (Satoh et al 2010). Functional analysis of sil1 and cyclophilin A in *An. campestris*-like mosquito should be carried out as they were major proteins in the female salivary glands.

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

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

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

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

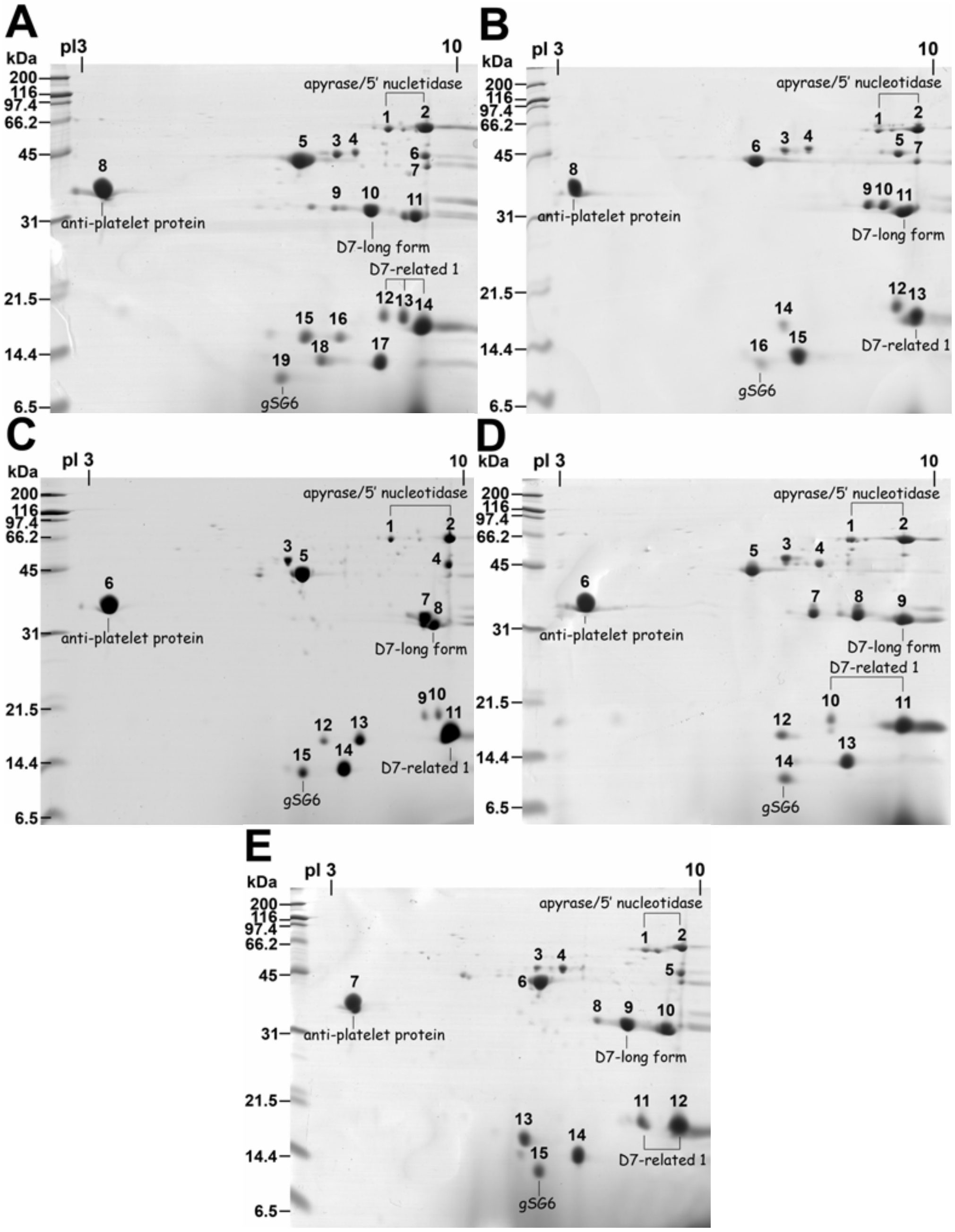


Fig. 1 2-DE analysis of female salivary glands of *An. barbirostris* complex. Proteins were separated on Immobiline DryStrips 7 cm, pH 3-10. Separation in the second dimension was performed using 15% SDS-PAGE. The gels were stained with Coomassie blue. Numbers indicate major salivary gland proteins.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

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ผลที่ได้รับจากโครงการ (output)

ผลที่ได้รับจากโครงการ (output):

1. มีผลงานวิจัยที่ได้ตีพิมพ์เผยแพร่แล้วในวารสารระดับนานาชาติ 2 เรื่อง และกำลังรอผลการพิจารณาจากวารสารที่ส่งตีพิมพ์ 1 เรื่อง ได้แก่

1.1 **Jariyapan* N**, Baimai V, Poovorawan Y, Roytrakul S, Saeung A, Thongsahuan S, Suwannamit S, Otsuka Y, Choochote W. (2010) Analysis of female salivary gland proteins of the *Anopheles barbirostris* complex (Diptera: Culicidae) in Thailand. Parasitol Res 107:509–516. (Impact factor 2011 = 2.149) (เอกสารแนบหมายเลข 1)

1.2 **Jariyapan N***, Roytrakul S, Paemanee A, Saeung A, Thongsahuan S, Sor-suwan S, Phattanawiboon B ,Poovorawan Y, Choochote W. (2012) Proteomic analysis of salivary glands of female *Anopheles barbirostris* species A2 (Diptera: Culicidae) by two-dimensional gel electrophoresis and mass spectrometry. Parasitol Res 111:1239–1249. (Impact factor 2011 = 2.149) (เอกสารแนบหมายเลข 2)

1.3 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). (เอกสารแนบหมายเลข 3)

* = correspondence author

2. ได้นำเสนอผลงานแบบโปสเตอร์ในการประชุมวิชาการนานาชาติ 3 เรื่อง

2.1 เรื่อง “Analysis of salivary gland proteins in *Anopheles barbirostris* complex (Diptera: Culicidae) in Thailand” ในการประชุมวิชาการ “การประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 9” ที่ โรงแรมฮอลิเดย์อินน์ รีสอรททรีเจนท์ บีช ชะอำ จ.เพชรบุรี วันที่ 15-17 ตุลาคม พ.ศ. 2552 (เอกสารแนบหมายเลข 4)

2.2 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 (เอกสารแนบหมายเลข 5) (เป็นส่วนหนึ่งของงานวิจัยของนักศึกษาคปก. นางสาวศรีวตาภรณ์ ส.สุวรรณ์ Grant no. PHD/0149/2551)

2.3 เรื่อง “Two-dimensional gel analysis of salivary gland proteins from female *Anopheles barbirostris* species A2 mosquitoes (Diptera: Culicidae)” ในการประชุมวิชาการ “การประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 10” ที่ โรงแรมฮอลิเดย์อินน์ รีสอร์ทรีเจนท์ บีช ชะอำ จ.เพชรบุรี วันที่ 14-16 ตุลาคม พ.ศ. 2553 (เอกสารแนบหมายเลข 6)

3. ผลงานวิจัยส่วนที่เหลือ เป็นข้อมูลพื้นฐานทางด้านอณูชีววิทยา สำหรับการนำไปใช้ศึกษาบทบาทที่เป็นไปได้ของผลผลิตจากต่อมน้ำลายของยุงก้นปล่องชนิดซับซ้อน *An. barbirostris* complex ที่เกี่ยวข้องกับการกินเลือดและการใช้แยกชนิดของยุงกลุ่มนี้

4. ได้สนับสนุนการเรียนการสอนระดับปริญญาเอก แก่นักศึกษาคปก จำนวน 2 คน คือ นางสาวศรีวตราภรณ์ ส.สุวรรณ์ (PHD/0149/2551) และ นางสาวเบญจรัตน์ พัฒนวิบูลย์ (PHD/0350/2552)

ลงนาม…………………..………………..
(รองศาสตราจารย์ ดร.นริศรา จริยะพันธุ์)
(หัวหน้าโครงการ)
วันที่ 25 กันยายน 2555

ภาคผนวก

เอกสารแนบหมายเลข 1

Jariyapan N, Baimai V, Poovorawan Y, Roytrakul S, Saeung A, Thongsahuan S, Suwannamit S, Otsuka Y, Choochote W. (2010) Analysis of female salivary gland proteins of the *Anopheles barbirostris* complex (Diptera: Culicidae) in Thailand. Parasitol Res 107:509–516.

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ORIGINAL PAPER

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

Narissara Jariyapan · Visut Baimai · Yong Poovorawan · Sittiruk Roytrakul · Atiporn Saeung · Sorawat Thongsahuan · Sittiporn Suwannamit · Yasushi Otsuka · Wej Choochote

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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.

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

N. Jariyapan (✉) · A. Saeung · S. Thongsahuan ·
S. Suwannamit · W. Choochote
Department of Parasitology, Faculty of Medicine,
Chiang Mai University,
Chiang Mai 50200, Thailand
e-mail: narsuwan@mail.med.cmu.ac.th

V. Baimai
Department of Biology and Center for Vectors and Vector-Borne Diseases, Faculty of Science,
Mahidol University,
Rama VI Road,
Bangkok 10400, Thailand

Y. Poovorawan
Department of Pediatric, Faculty of Medicine,
Chulalongkorn University,
Bangkok 10330, Thailand

S. Roytrakul
National Center for Genetic Engineering and Biotechnology (BIOTEC),
National Science and Technology Development Agency,
Pathumthani 12120, Thailand

Y. Otsuka
Department of Infectious Disease Control, Faculty of Medicine,
Oita University,
Hasama, Oita 879-5593, Japan

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

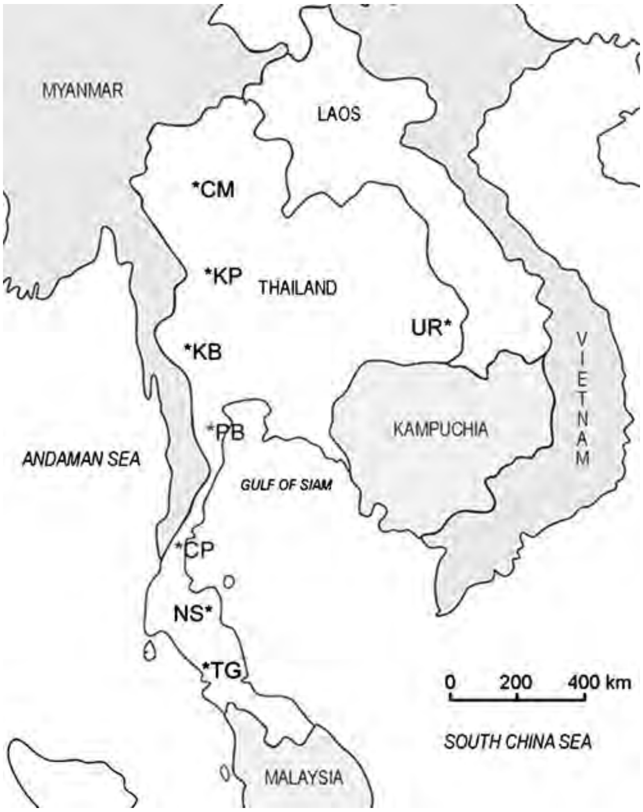

Fig. 1 Collection sites (*) for the *A. barbirostris/campestris* group in Thailand. *CM* Chiang Mai, *KP* Kamphaeng Phet, *KB* Kanchanaburi, *UR* Ubon Ratchathani, *PB* Phetchaburi, *CP* Chumphon, *NS* Nakhon Si Thammarat, *TG* Trang

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′-TGTGAACTGCAGGACACAT-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 nanocolumn (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

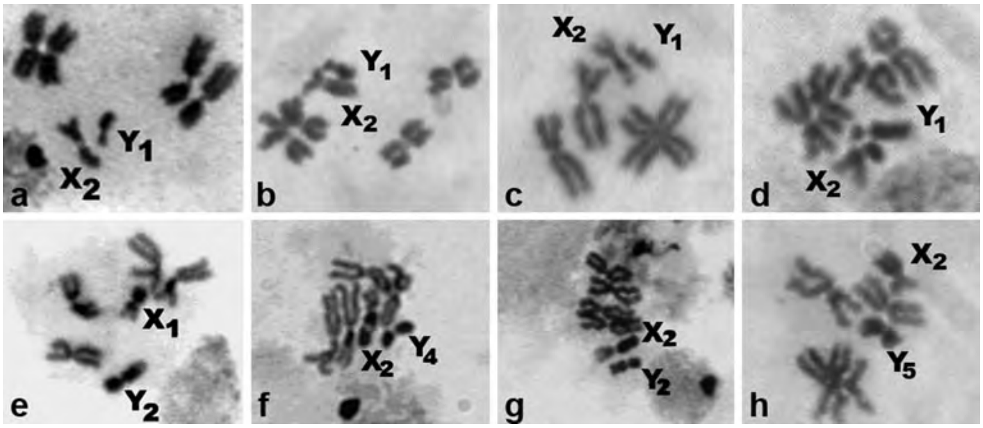

Fig. 2 Metaphase karyotypes of *A. barbirostris* complex. **a** Species A1, Form A (X_2, Y_1: Chiang Mai); **b** species A2, Form A (X_2, Y_1: Phetchaburi); **c** species A3, Form A (X_2, Y_1: Kanchaburi); **d** species A4, Form A (X_2, Y_1: Chiang Mai); **e** species A1, Form B (X_1, Y_2: Chumphon); **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)

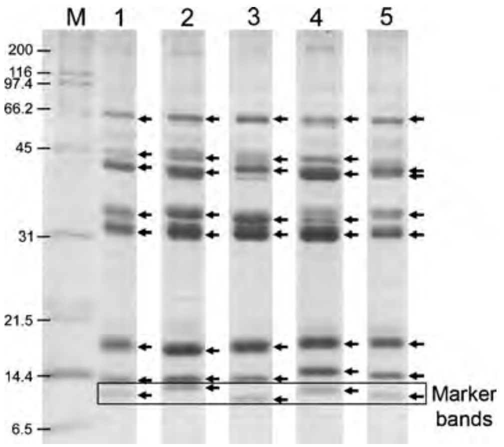

Fig. 3 Electrophoretic profiles of salivary glands obtained from female *A. barbirostris* complex. Proteins of one salivary gland pair were separated on 15% SDS–polyacrylamide gels and stained with Comassie blue. *Lane 1 A. barbirostris* species A1, *lane 2* species A2, *lane 3* species A3, *lane 4*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

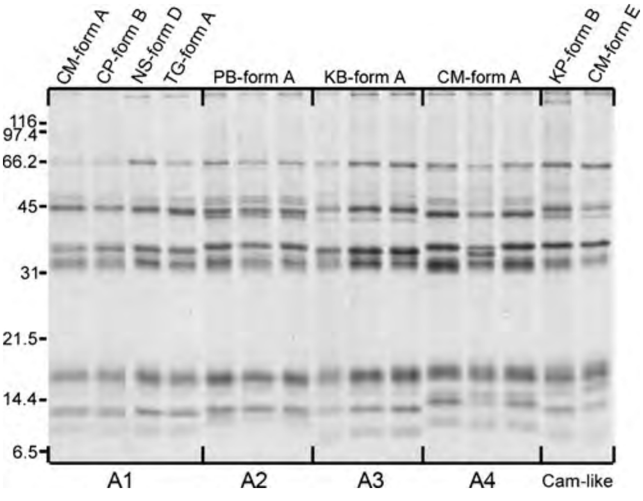

Fig. 4 Electrophoretic protein profiles of salivary glands obtained from female *A. barbirostris* species A1, A2, A3, and A4 and *A. campestris*-like. Proteins of one pair of salivary gland were separated on a 15% SDS–polyacrylamide gel and stained with Comassie blue. Mosquitoes from *CM* Chiang Mai, *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 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.

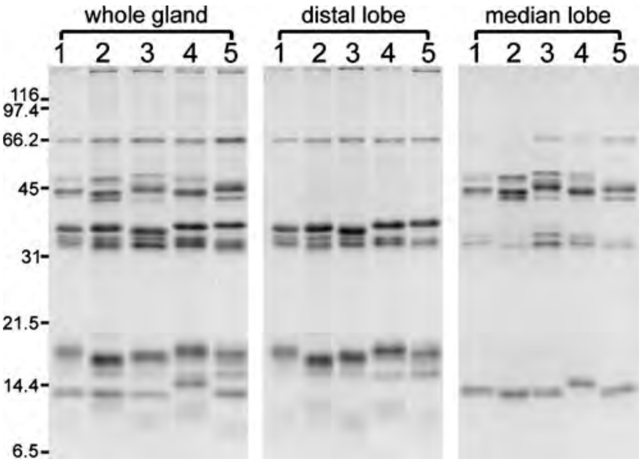

Fig. 5 Electrophoretic protein profiles of salivary glands obtained from female *A. barbirostris* species A1, A2, A3, and A4 and *A. campestris*-like. Proteins of one pair of whole salivary gland or distal lobe or median lobe were separated on a 15% SDS–polyacrylamide gel and stained with Comassie blue. *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

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*

Narissara Jariyapan, Sittiruk Roytrakul, Atchara Paemanee, Anuluck Junkum, Atiporn Saeung, Sorawat Thongsahuan, Sriwatapron Sor-suwan, et al.

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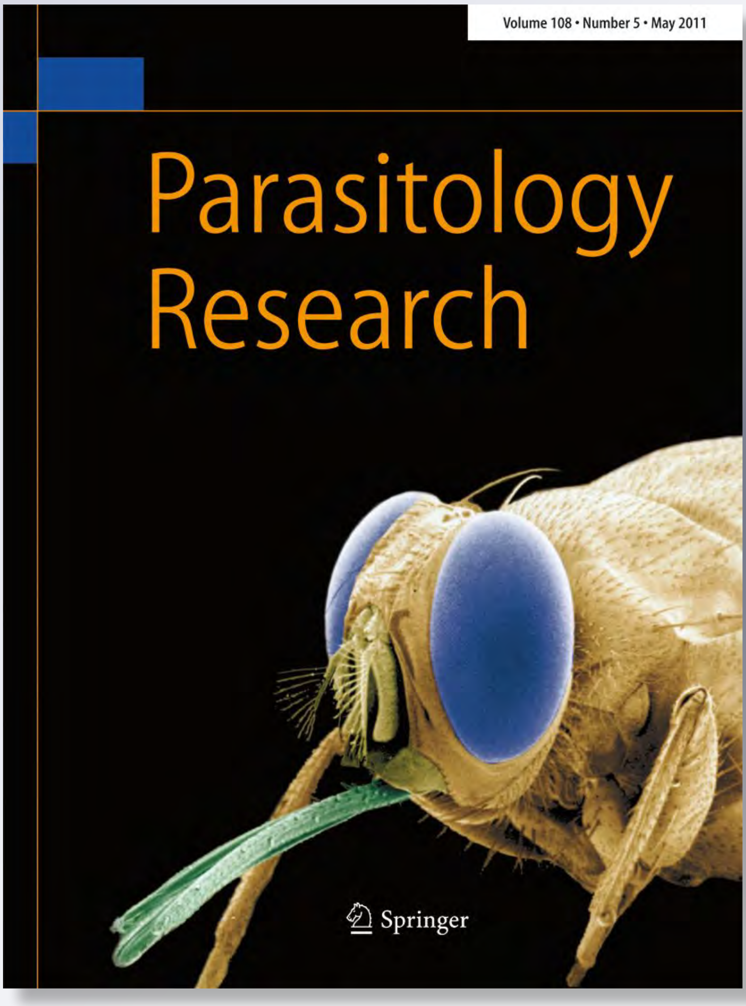

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ORIGINAL PAPER

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

Narissara Jariyapan · Sittiruk Roytrakul · Atchara Paemanee · Anuluck Junkum · Atiporn Saeung · Sorawat Thongsahuan · Sriwatapron Sor-suwan · Benjarat Phattanawiboon · Yong Poovorawan · Wej Choochote

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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.

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

N. Jariyapan (✉) · A. Junkum · A. Saeung · S. Thongsahuan · S. Sor-suwan · B. Phattanawiboon · W. Choochote
Department of Parasitology, Faculty of Medicine,
Chiang Mai University,
Chiang Mai 50200, Thailand
e-mail: narsuwan@mail.med.cmu.ac.th

S. Roytrakul · A. Paemanee
National Center for Genetic Engineering and
Biotechnology (BIOTEC),
National Science and Technology Development Agency,
Pathumthani 12120, Thailand

Y. Poovorawan
Department of Pediatrics, Faculty of Medicine,
Chulalongkorn University,
Bangkok 10330, Thailand

related to blood feeding are synthesized by the medial and the distal regions of the lateral lobes (James 2003). Also, salivary gland invasion of malaria parasites is limited to the 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 nanocolumn (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

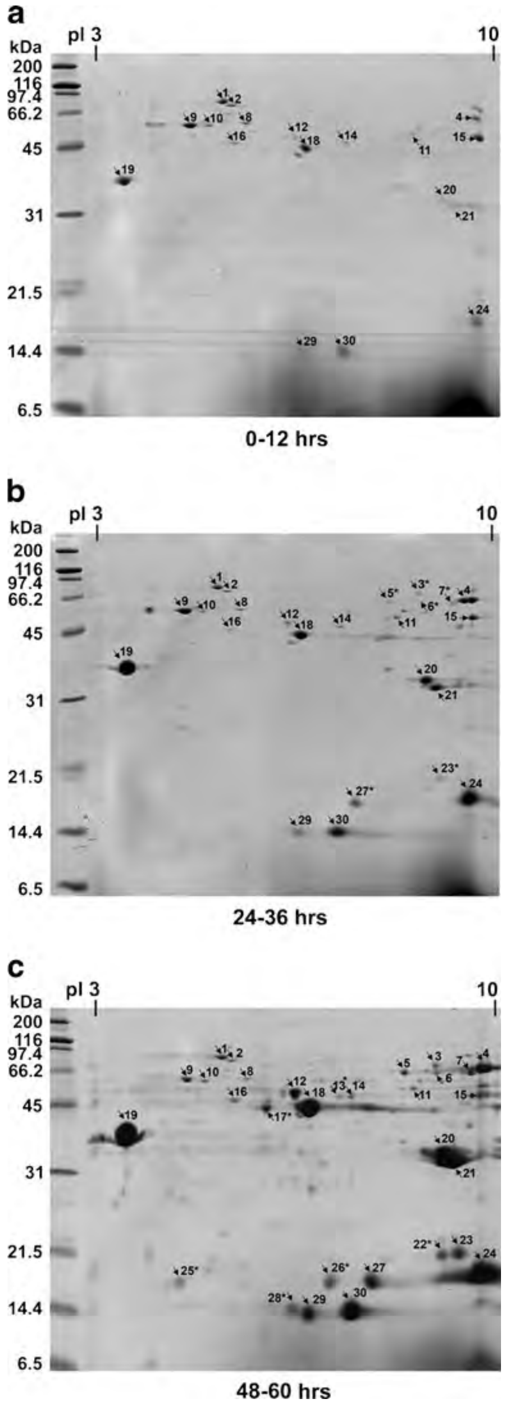


Fig. 1 Two-dimensional gel analysis of female salivary gland proteins of *A. barbirostris* species A2 mosquitoes according to age. Proteins were separated on Immobiline DryStrips 7 cm, pI 3–10. Separation in the second dimension was performed using 15 % SDS-PAGE. The gels were stained with Coomassie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

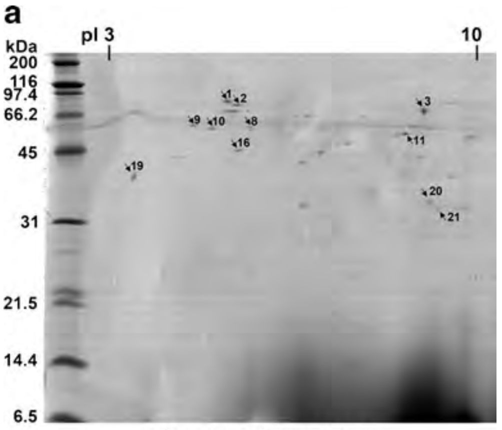
[e] 24–36 h post emergence

[f] 48–60 h post emergence

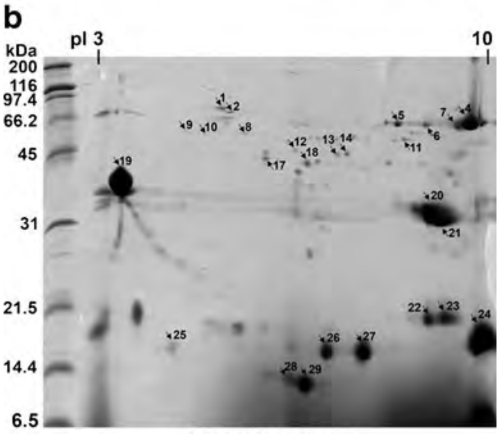
[g] Index: relative volume expressed on the hour when the relatively average normalization volume of protein was expressed at the first hour

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).

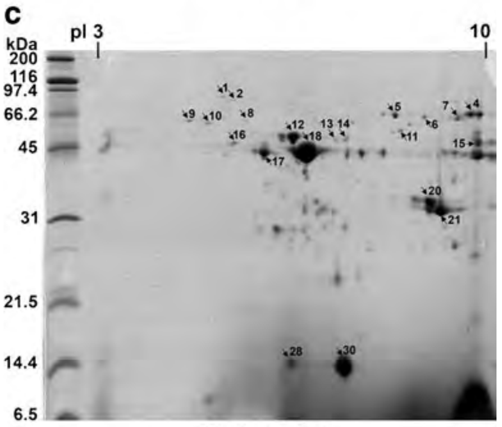
Apyrases are nucleoside triphosphate-diphosphohydrolases present in a variety of organisms. In *A. aegypti*, the salivary apyrase was identified as a member of the 5′ nucleotidase family (Champagne et al. 1995). Recently, apyrase of *Aedes albopictus* was cloned and characterized (Dong et al. 2012). 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



Proximal-lateral lobes



Distal-lateral lobes



Median lobes

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

Jariyapan, N.[1]*, Baimai, V.[2], Poovorawan, Y.[3], Roytrakul, S.[4], Thongsahuan, S.[1], Saeung, A.[1], Suwannamit, S.[1], Takaoka, H.[5], Choochote, W.[1]

[1]Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.
[2]Department of Biology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok, Thailand.
[3]Department of Peditric, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
[4]Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Pathumthani, Thailand.
[5]Department of Infectious Disease Control, Faculty of Medicine, Oita University, Hasama, Oita, Japan.

***Corresponding author.** Tel.:0-5394-5343 ext. 504; Fax:0-5321-7144. E-mail: narsuwan@mail.med.cmu.ac.th

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).

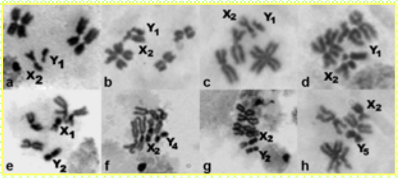

Fig. 1 Metaphase karyotypes of *A. barbirostris* complex. **(a)** species A1, Form A (X2, Y1: Chiang Mai); **(b)** species A2, Form A (X2, Y1: Phetchaburi); **(c)** species A3, Form A (X2, Y1: Kanchaburi); **(d)** species A4, Form A (X2, Y1: Chiang Mai); **(e)** species A1, Form B (X1, Y2: Chumphon); **(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[a]	Karyotypic form (X,Y)	Length of ITS2 (base pair)	Species
Chiang Mai (18°47′N, 98° 59′E)	aCMA1	A (X_2,Y_1)	1,822	*An. barbirostris*-A1
	aCMA2	A (X_2,Y_1)	1,637	*An. barbirostris*-A4
	hCME3	E (X_2,Y_5)	1,612	*An. campestris*-like
	hCME4	E (X_2,Y_5)	1,612	*An. campestris*-like
Kamphaeng Phet (16°28′N, 99° 31′E)	aKPB1	B (X_2,Y_2)	1,612	*An. campestris*-like
Kanchanaburi (14°01′N, 99° 32′E)	aKBA2	A (X_2,Y_1)	1,031	*An. barbirostris*-A3
Ubon Ratchathani (15°15′N, 104° 52′E)	aUBA7	A (X_2,Y_1)	1,822	*An. barbirostris*-A1
Phetchaburi (13°09′N, 100° 04′E)	aPBA3	A (X_2,Y_1)	1,678	*An. barbirostris*-A2
Chumphon (10°29′N, 99° 11′E)	aCPB4	B (X_1,Y_2)	1,822	*An. barbirostris*-A1
Nakhon Si Thammarat (08°32′N, 99° 57′E)	aNSD1	D (X_2,Y_4)	1,822	*An. barbirostris*-A1
Trang (07°31′N, 99° 37′E)	aTGA10	A (X_2,Y_1)	1,822	*An. barbirostris*-A1

[a]: h – human bait, a – animal bait

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.

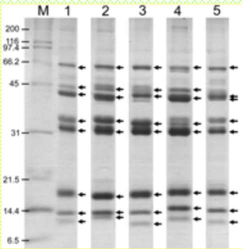

Fig. 2 Electrophoretic profiles of salivary glands obtained from female *A. barbirostris* complex. Proteins of one salivary gland pair were separated on 15% SDS-polyacrylamide gels and stained with Comassie blue. Lane 1, *An. barbirostris* species A1; lane 2, species A2; lane 3, species A3; lane 4, species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

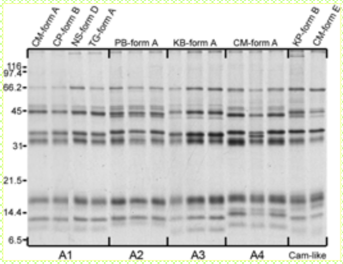

Fig. 3 Electrophoretic profiles of salivary glands obtained from female *An. barbirostris* A1, *An. barbirostris* A2, *An. barbirostris* A3, *An. barbirostris* A4, and *An. campestris*-like. Proteins of one pair of salivary gland were separated on a 15% SDS-polyacrylamide gel and stained with Comassie 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

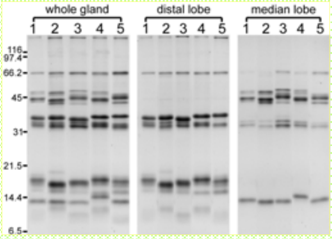

Fig. 4 Electrophoretic profiles of salivary glands obtained from female *An. barbirostris* A1, *An. barbirostris* A2, *An. barbirostris* A3, *An. barbirostris* A4, and *An. campestris*-like. Proteins of one pair of whole salivary gland or distal lobe or median lobe were separated on a 15% SDS-polyacrylamide 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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Two-dimensional gel analysis of salivary gland proteins from female *Anopheles barbirostris* species A2 mosquitoes (Diptera: Culicidae)

**Jariyapan, N.[1]*, Choochote, W.[1], Roytrakul, S.[2], Saeung, A.[1], Thongsahuan, S.[1],
Sor-suwan, S.[1], Ta-ai, K.[1], Poovorawan, Y.[3], Baimai, V.[4]**

[1]*Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
[2]*National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Pathumthani, Thailand*
[3]*Department of Pediatric, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*
[4]*Department of Biology and Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, Bangkok, Thailand*

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.

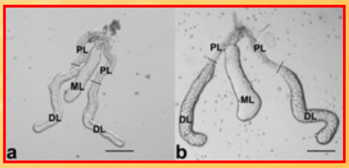

Fig. 1 Representative female salivary glands of *A. barbirostris* species A2. a: a salivary gland from female 1 hr post emergence; b: a salivary gland from female 72 hr post emergence showing proximal region of the lateral lobe (PL), distal region of the lateral lobe (DL), and median lobe (ML). Bar represents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

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A. Saeung , V. Baimai, Y. Otsuka, et al. *Parasitol Res.* 102, 499 (2008)
N. Jariyapan, V. Baimai, Y. Poovorawan, et al. *Parasitol Res.* 107, 509 (2010).
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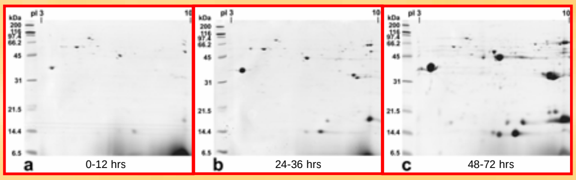
Acknowledgments

This work was supported by the Thailand Research Fund (RMU5180011 to NJ), the Thailand Research Fund through the Royal Golden Jubilee Ph.D. program (PHD/0149/2551) and the Faculty of Medicine Endowment Fund, Chiang Mai University.

Results

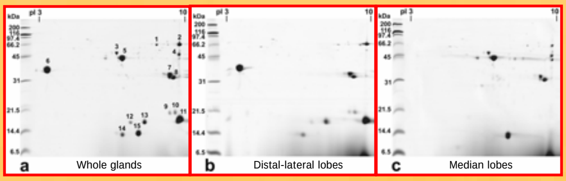
1. Age-dependence of the salivary gland protein profiles (Fig. 2)



0-12 hrs 24-36 hrs 48-72 hrs

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)



Whole glands Distal-lateral lobes Median lobes

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)

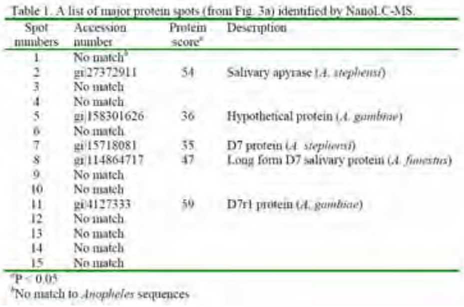


Table 1. A list of major protein spots (from Fig. 3a) identified by NanoLC-MS.

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.

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]

[a]Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
[b]Department of Biology and Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
[c]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Pathumthani 12120, Thailand
[d]Department of Pediatric, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

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

N. Jariyapan, V. Baimai, Y. Poovorawan Y, et al. Parasitol Res. 107, 509 (2010).
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)

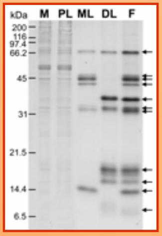


Fig. 2 Female and male salivary gland proteins of *An. campestris*-like. Salivary gland proteins were separated on 15% SDS- polyacrylamide gels and stained with Coomassie blue. M: sixty male salivary glands; PL: fifty female proximal-lateral lobes; ML: two median lobes; DL: two distal-lateral lobes;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

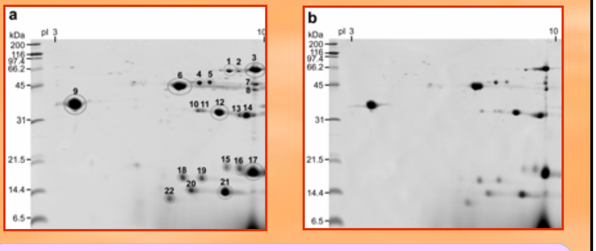


Fig. 3 Coomassie blue-staied 2D gels of female salivary glands of *An. campestris*-like. Numbers indicate major salivary gland proteins. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. a: a representaive of two-dimensional gels of non blood-fed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

4. NanoLC-MS analysis (Table 1)

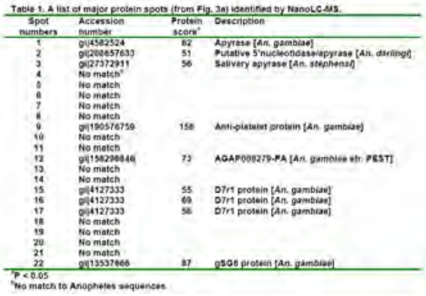


Table 1. A list of major protein spots (from Fig. 3a) identified by NanoLC-MS.

Spot numbers	Accession number	Protein score[a]	Description
1	gi|4582524	82	Apyrase [*An. gambiae*]
2	gi|208657633	51	Putative 5'nucleotidase/apyrase [*An. darlingi*]
3	gi|27372911	56	Salivary apyrase [*An. stephensi*]
4	No match[b]		
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|158298846	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	58	D7r1 protein [*An. gambiae*]
18	No match		
19	No match		
20	No match		
21	No match		
22	gi|13537666	87	gSG6 protein [*An. gambiae*]

[a] $P < 0.05$
[b] 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.