# CHAPTER III

## Results

### 3.1 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 three and five days was approximately 0.1 ± 0.05 µg/male and female was 1.38 ± 0.01 µg/female (n=25).

### 3.2 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. 3.1. 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).

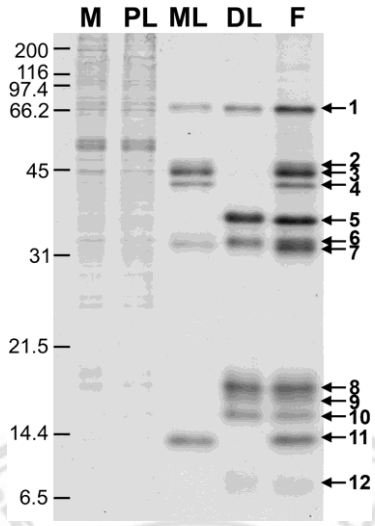


**Figure 3.1** Female and male salivary gland protein profiles of *An. campestris*-like. The salivary gland proteins were separated on 15 % SDS-polyacrylamide gels and stained with CBB. M: sixty male salivary glands; PL: fifty female proximal-lateral lobes; ML: two median lobes; DL: two distal-lateral lobes; F: two whole female salivary glands. Molecular mass markers are indicated on the left in kilodalton. Arrows indicate major salivary gland proteins of female mosquitoes.

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 (Figure. 3.2a). The gel (Figure. 3.2a) was considered the standard reference gel. The molecular mass of these spots varied from 10 to 72 kDa, with pI ranging of 3.9-10. Nineteen major protein spots were detected in the female mosquitoes and identified by nanoLC-MS. Spot numbers in Table 3.1 correspond to the salivary gland proteins shown in Figure. 3.2a. From the 19 major protein spots, 14 spots are positive for glycoprotein staining (Figure. 3.2b and Table 3.1).

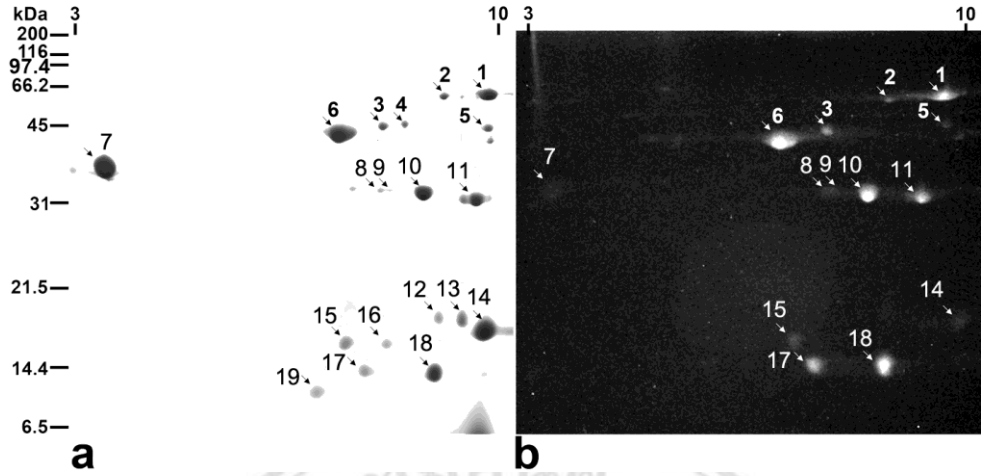


**Figure 3.2** Two-dimensional gel analysis of female salivary gland proteins of *An. campestris*-like. Proteins were separated on Immobiline DryStrips 7 cm, pI 3–10. Separation in the second dimension was performed using 15 % SDS-PAGE. Molecular mass markers are indicated on the left in kilodalton. 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.

Expression patterns of the protein spots in male and different lobes of female salivary gland are shown in Figure 3.3 and Table 3.1. Ten major protein spots (SN1, 2, 3, 4, 6, 7, 10, 11, 14, and 18) were similarly found with weak intensity in the male salivary glands (Figure 3.3a) and the proximal-lateral lobes of female glands (Figure 3.3b). Five major protein spots (SN12, 13, 15, 16, and 19) were detected only in the distal-lateral lobes (Figure 3.3c) whereas three major spots (SN5 and 17) were specific in the medial lobe (Figure 3.3d).

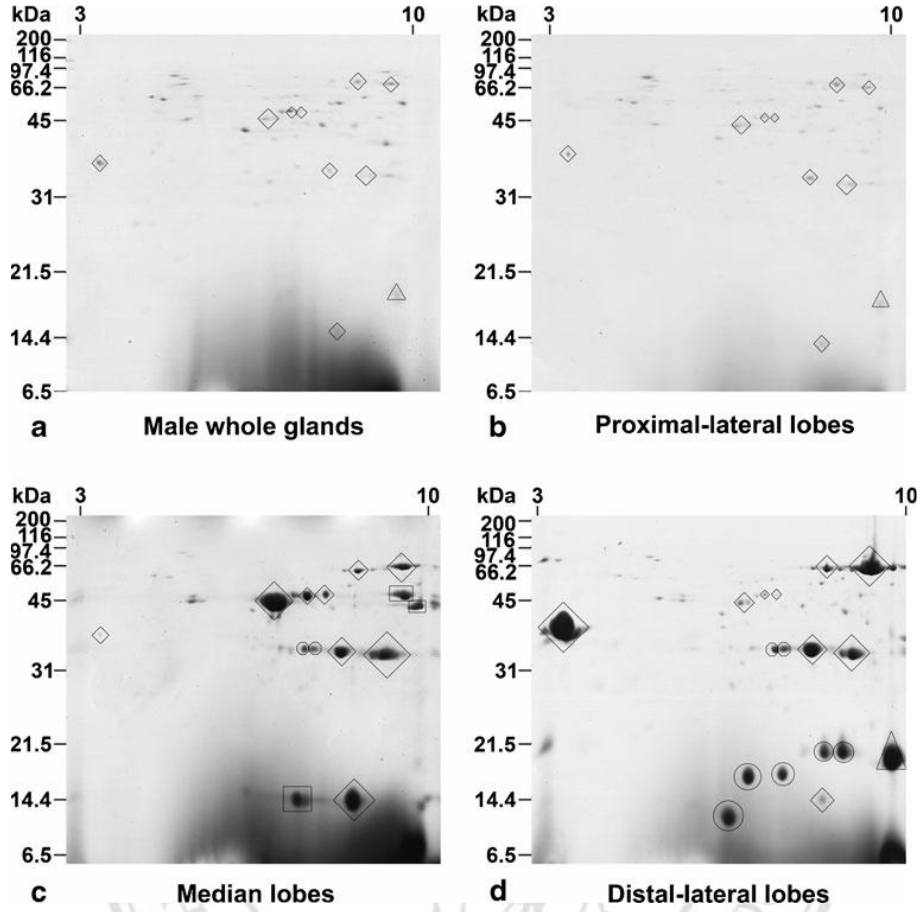


**Figure 3.3** Two-dimensional gel analysis of proteins expressed in male and different regions of female *An. campestris*-like salivary glands. Proteins were separated on Immobiline DryStrips 7 cm, pI 3-10. Separation in the second dimension was performed using 15 % SDS-PAGE. The gels were stained with CBB. Molecular mass markers are indicated on the left in kilodalton. 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. Circle, a major spot found in distal-lateral lobe, square, a major spot found in medial lobe, triangle, a major spot found in male, proximal-lateral and distal-lateral lobes, octagon, a major spot found in medial and distal-lateral lobes, diamond, a major spot found in male and all female lobes.

### 3.3 Comparison of Female Salivary Gland Protein Profiles between Unfed and Blood Fed *An. campestris*-like Mosquitoes

The experimental design was describe in Chapter II (Figure 2.1), each experiment yielding five groups of mosquitoes, and each group containing 70 individuals: sugar-fed, taken after one blood meal, control for one blood meal, taken after two blood meals, and control for two blood meals. This experiment was performed three times, each experiment produced essentially identical results, and representative results from one experiment are shown in Figure 3.4. Analysis by 2-DE showed that female *An. campestris*-like salivary glands contained 19 major proteins (Figure. 3.4a). The molecular mass of these well-resolved spots varied from 10-72 kDa, with pI ranging from 3.9-10. To identify them, each major spot was excised, digested with trypsin, and subjected to NanoLC-MS analysis (Table 3.1). Sixteen of the 19 spots could be assigned an identity, these including apyrase, which was spot number 1 (SN1), 5'-nucleotidase/apyrase (SN2), anti-platelet protein (SN7), D7 (SN10), D7-related 1 (SN12), and gSG6 (SN19). Comparing the protein profiles of female salivary glands of the unfed controls (Figure. 3.4a) and the blood fed mosquitoes from the first blood meal group (Figure. 3.4b) they were essentially identical regarding the number of proteins detected. However, there were significant differences in the density of the protein spots, in the blood fed mosquitoes the density of all the major protein spots was reduced compared to the unfed controls. A heat shock cognate 70 kDa protein of *Ae. aegypti* (accession number gi|94468966) was used as an internal control in 2-DE gels (Table 3.1, 3.2, Figure. 3.4). This protein is circled in Figure 3.4a-e and showed no significant difference in density between samples. To provide a quantitative measure of depletion in the major proteins, gel imaging analysis was performed (Table 3.2). The amount of protein depletion varied from spot to spot (0.13-7.15) and whilst these values are not directly comparable due to potential differences in the staining of individual proteins, they do suggest differential delivery of salivary gland proteins during blood feeding. This was supported by analysis of the % of each protein depleted, revealing a range of values from SN2, which showed the highest depletion at 79.71%, to SN14, which showed 13.18% depletion. However, excepting SN6, 14, and 19, the remaining 16 proteins showed depletion of approximately 50% or greater. Thus the taking of one

blood meal significantly depleted the salivary gland contents, but individual proteins were depleted to differing extents.

### 3.4 Comparison of Female Salivary Gland Protein Profiles After One Blood Feed but Before Second Blood Feed

After taking one blood meal, the salivary gland protein content was replenished over the next 14 days, with 17 of the 19 major proteins increasing in amount again (Figure. 3.4c, Table 3.2). The two exceptions were SN4 (a conserved hypothetical protein) and SN16 (unidentified), which were not detected in mosquitoes prior to the second blood meal. Therefore these two components, which were depleted after one feed, did not recover but disappeared from the salivary glands, at least as major components. It should also be noted that they were absent from the sugar-fed control group (Figure. 3.4e), which was age matched for the second blood meal groups, indicating this may be age-related decline. After comparing the data from unfed three to four days old mosquito salivary glands with the glands of mosquitoes before a second blood feeding, all major protein spots were increased significantly. Similarly, when comparing this data with unfed 17-18 days old mosquito salivary glands, most of major protein spots were increased significantly, except SN 13, which was decreased significantly. Regarding the remaining majority of proteins that did increase significantly, in most cases these increases were such that the amounts present immediately before taking a second meal were actually much greater than before taking the first blood meal. For example, apyrase (SN1) had an average value of 4.16 before the first meal and 8.62 before the second meal (~2 fold difference), and D7-related 1 (SN12) with a value of 0.4 before the first meal and 4.95 before the second meal (~12 fold difference). Exceptions to these increases were SN7 (anti-platelet protein) and SN11, which were at slightly lower values, although still significantly replenished following the first blood meal.

### 3.5 Comparison of Female Salivary Gland Protein Profiles Before and After a Second Blood Feed in *Anopheles campestris*-like Mosquitoes

Comparison of the amount of each of the 17 major proteins present before and after the second blood meal again revealed statistically significant depletion of most

protein spots, the exceptions being SN11, 15, and 18. Therefore, the remaining 14 salivary gland proteins are those predicted to accompany sporozoites from an infected mosquito. However, there were significant variations in the depletion of individual proteins comparing first and second blood meals. For example, SN11 showed a 67% depletion after the first blood meal, but only a 3% depletion after the second meal. For most proteins the % depletion after the second meal was lower than after the first meal, although for SN6, 13, 14, and 19 it was higher. However, because individual proteins were replenished to different extents following the first blood meal, these % values do not necessarily reflect the absolute amounts of protein delivered. For example, the amount of apyrase (SN1) depleted after the first meal (2.38) was similar to after the second meal (2.63). In contrast, the amount of anti-platelet protein (SN7) depleted was more after the first meal (7.15) compared to the second meal (2.54), whereas the amount of D7-related 1 (SN12) was less after the first meal (0.22) compared to the second (2.21). Overall these results indicate not only differential depletion and, therefore, delivery of individual salivary gland proteins within a blood feed, but also between first and second blood feeds.

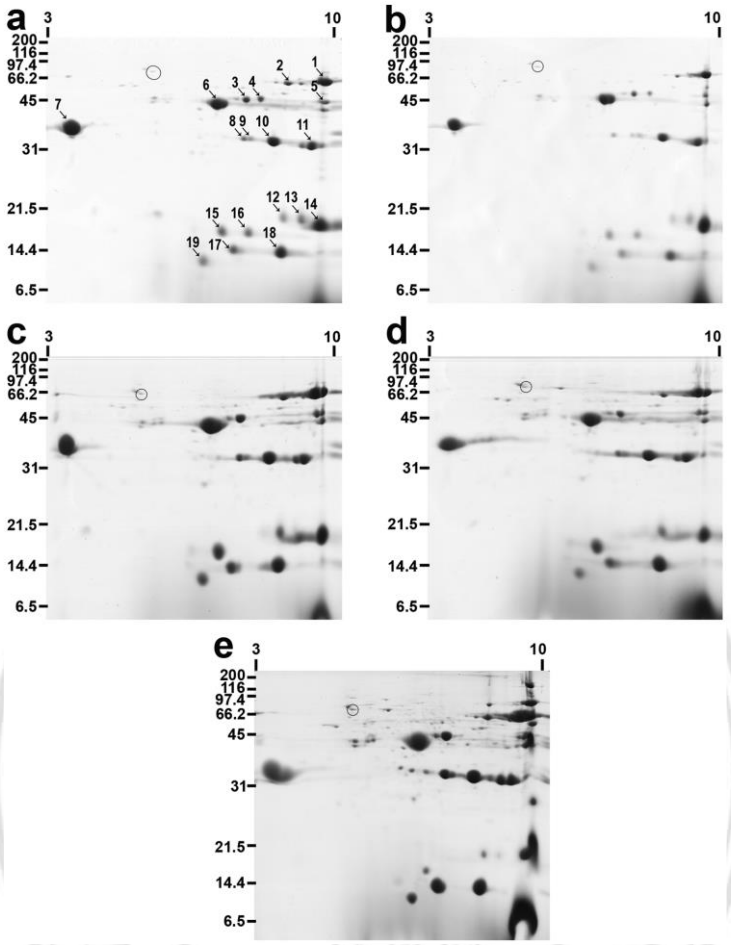


**Figure 3.4** Comparison of 2-DE protein profiles of female salivary gland proteins of *An. campestris*-like. Proteins were separated on Immobiline Dry Strips 7 cm, pI 3–10. Separation in the second dimension was performed using 15% SDS-PAGE. The gels were stained with Coomassie blue. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. Numbers indicate major salivary gland proteins. a representative of 2-DE gels of salivary gland proteins of unfed females from the first blood meal group; b salivary gland proteins of blood fed females the first blood meal group; c salivary gland proteins of unfed females from the second blood meal group; d salivary gland proteins of blood-fed females the second blood meal group; e salivary gland proteins of unfed females from sugar fed control group. Circle indicates an internal control protein.

**Table 3.1** A list of major protein spots significantly decreased in volume immediately after the first blood feeding in female *An. campestris*-like salivary glands identified by NanoLCMS.

| SN[a] | Accession number[b] | Protein Description [species] | Protein score[d] | No. of peptides/ %coverage | Database Mw/pI | Observed Mw/pI | Peptide Sequences | Expression pattern | Glycoprotein | Classification |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | gi\|4582524 | apyrase [*An. gambiae*] | 31 | 1/3 | 62.1/8.9 | 68/9.8 | R.VFHTVQELR.K | M, PL, DL, ML | Yes | Blood feeding |
| 2 | gi\|208657633 | putative 59-nucleotidase/apyrase [*An. darlingi*] | 68 | 2/6 | 63.5/8.7 | 67/8.0 | K.NVPDQSFPLTLIHINDLHAR.F<br>K.QLPPDAMTLGNHEFDHSPK.G | M, PL, DL, ML | Yes | Blood feeding |
| 3 | gi\|347968035 | AGAP002538-PA [*An. gambiae* str. PEST] | 37 | 1/2 | 36.5/5.5 | 56/7.5 | R.NLTTDELSR.L | | Yes | |
| 4 | gi\|170033361 | conserved hypothetical protein [*Cx. quinquefasciatus*] | 41 | 1/2 | 38.6/5.6 | 55/7.2 | R.NDDLHDIER.F | | | |
| 5 | gi\|242021351 | hypothetical protein Phum_PHUM512530 [*Pediculus humanus corporis*] | 35 | 1/3 | 33.7/9.4 | 55/9.8 | R.DLNLLTPTSM.- | ML | Yes | Unknown |
| 6 | gi\|170046888 | serine/threonine-protein kinase rio3 [*Cx. quinquefasciatus*] | 33 | 2/5 | 56.9/9.4 | 52/6.7 | R.SRLSGAASCDHR.S<br>R.VGYKVNEDGEMVTK.H | M, PL, DL, ML | Yes | Housekeeping |
| 7 | gi\|190576759 | anti-platelet protein [*An. gambiae*] | 40 | 1/4 | 27.2/4.1 | 38/4.1 | R.EQELSDCIVDKR.D | M, PL, ML, DL | Yes | Blood feeding |
| 8 | NSH | - | - | - | - | 37/7.1 | - | | Yes | |
| 9 | gi\|241998444 | sil1, putative [*Ixodes scapularis*] | 35 | 1/2 | 36.4/5.1 | 37/7.3 | R.LNLETGRR.E | ML, DL | Yes | Housekeeping |
| 10 | gi\|15718081 | D7 protein [*An. stephensi*] | 41 | 1/3 | 36.9/8.8 | 36/7.7 | R.QLYHGTVEGAAK.I | M, PL, ML, DL | Yes | Blood feeding |
| 11 | gi\|158285343 | AGAP007618-PA [*An. gambiae* str. PEST] | 37 | 1/2 | 30.9/8.4 | 35/9.4 | R.LADMMR.Q | M, PL, ML, DL | Yes | Unknown |
| 12 | gi\|4538887 | D7-related 1 protein [*An. gambiae*] | 55 | 1/6 | 19.1/9.2 | 20/7.9 | K.LIKPLNAIEK.D | DL | No | Blood feeding |
| 13 | gi\|241616200 | cyclophilin A, putative [*I. scapularis*] | 41 | 1/4 | 22.1/9.2 | 20/9.0 | K.FEDENFILK.H | DL | No | Housekeeping |

**Table 3.1** (continued)

| SN[a] | Accession number[b] | Protein Description [species] | Protein score[d] | No. of peptides/ %coverage | Database Mw/pI | Observed Mw/pI | Peptide Sequences | Expression pattern | Glycoprotein | Classification |
|---|---|---|---|---|---|---|---|---|---|---|
| 14 | gi\|16225961 | short form D7r1 salivary protein [*An. arabiensis*] | 52 | 1/6 | 19/9.2 | 19/9.8 | K.LIKPLNAIEK.D | M, PL, DL | Yes | Blood feeding |
| 15 | gi\|270014872 | hypothetical protein TcasGA2_ TC010859 [*Tribolium castaneum*] | 31 | 1/4 | 16.1/7.8 | 17/6.8 | -.MGDMQR.R | DL | Yes | Unknown |
| 16 | NSH | - | - | - | - | 17/7.3 | - | DL | No | - |
| 17 | NSH | - | - | - | - | 14/7.0 | - | ML | Yes | - |
| 18 | gi\|312381960 | hypothetical protein AND_05658 [*An. darlingi*] | 38 | 1/4 | 16.9/7.7 | 14/7.9 | R.KSLEAMR.E | M, PL, ML, DL | Yes | Unknown |
| 19 | gi\|13537666 | gSG6 protein [*An. gambiae*] | 87 | 2/10 | 13.7/5.3 | 12/6.5 | R.DKVYCGHLDCTR.V<br>K.VYCGHLDCTR.V | DL | No | Blood feeding |
| Control | gi\|94468966 | heat shock cognate 70 [*Aedes aegypti*] | 515 | 10/17 | 71.4/5.3 | 77.0/5.4 | R.TTPSYVAFTDTER.L<br>K.NQVAMNPTNTIFDAK.R<br>K.DAGTISGLNVLR.I<br>R.IINEPTAAAIAYGLDK.K<br>R.IINEPTAAAIAYGLDKK.T<br>R.FEELNADLFR.S<br>K.ASIHDIVLVGGSTR.I<br>K.LLQDFFNGK.E<br>K.FELSGIPPAPR.G<br>K.NALESYCFNMK.A | M, PL, ML, DL | Yes | Housekeeping |

[a]Spot number refers to those shown in Fig. 3.4a.

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

[c]NSH = not significant hit.

[d]Mowse score ≥30.

**Table 3.2** Amounts of depletion of major salivary gland proteins from unfed mosquitoes and blood fed to repletion on mice.

| SN[a] | Frist blood meal group | | | | Second blood meal group | | | |
|---|---|---|---|---|---|---|---|---|
| | ASD±SD[b] | | Amount depletion | %Depletion | ASD±SD | | Amount depletion | %Depletion |
| | Unfed | Blood fed | | | Unfed | Blood fed | | |
| 1 | 4.16±0.08 | 1.78±0.07 | 2.38 | 57.11[c] | 8.62±0.05 | 5.99±0.01 | 2.63 | 30.53[c] |
| 2 | 0.55±0.03 | 0.11±0.01 | 0.44 | 79.71[c] | 1.68±0.02 | 0.61±0.01 | 1.07 | 63.57[c] |
| 3 | 0.70±0.01 | 0.30±0.01 | 0.4 | 56.87[c] | 1.60±0.01 | 0.69±0.01 | 0.91 | 52.94[c] |
| 4 | 0.46±0.01 | 0.12±0.01 | 0.34 | 74.38[c] | (-)[d] | (-)[d] | (-)[d] | (-)[d] |
| 5 | 0.59±0.02 | 0.24±0.01 | 0.35 | 59.16[c] | 1.49±0.01 | 0.69±0.01 | 0.8 | 53.54[c] |
| 6 | 4.61±0.06 | 3.42±0.02 | 1.19 | 25.96[c] | 8.95±0.05 | 6.16±0.02 | 2.79 | 31.14[c] |
| 7 | 10.31±0.08 | 3.16±0.05 | 7.15 | 69.36[c] | 8.02±0.06 | 5.48±0.05 | 2.54 | 31.68[c] |
| 8 | 0.26±0.01 | 0.05±0.01 | 0.21 | 78.89[c] | 0.79±0.01 | 0.43±0.01 | 0.36 | 45.43[c] |
| 9 | 0.22±0.01 | 0.09±0.01 | 0.13 | 57.06[c] | 0.42±0.01 | 0.34±0.01 | 0.08 | 19.15[c] |
| 10 | 2.67±0.08 | 1.38±0.01 | 1.29 | 48.19[c] | 4.07±0.02 | 3.22±0.01 | 0.85 | 20.88[c] |
| 11 | 4.40±0.04 | 1.43±0.03 | 2.97 | 67.41[c] | 3.59±0.01 | 3.48±0.01 | 0.11 | 3.03 |
| 12 | 0.40±0.01 | 0.18±0.01 | 0.22 | 53.90[c] | 4.95±0.03 | 2.74±0.02 | 2.21 | 45.10[c] |
| 13 | 0.79±0.01 | 0.40±0.01 | 0.39 | 48.74[c] | 1.77±0.01 | 0.76±0.01 | 1.01 | 56.86[c] |
| 14 | 4.39±0.04 | 3.81±0.02 | 0.58 | 13.18[c] | 4.90±0.06 | 2.69±0.02 | 2.21 | 45.10[c] |
| 15 | 0.69±0.01 | 0.29±0.01 | 0.4 | 58.34[c] | 2.20±0.02 | 1.93±0.02 | 0.27 | 12.34 |
| 16 | 0.53±0.01 | 0.23±0.01 | 0.3 | 56.53[c] | (-)[d] | (-)[d] | (-)[d] | (-)[d] |
| 17 | 0.85±0.01 | 0.43±0.01 | 0.42 | 49.25[c] | 1.85±0.01 | 1.37±0.01 | 0.48 | 26.27[c] |
| 18 | 2.48±0.01 | 1.09±0.02 | 1.39 | 55.93[c] | 4.81±0.02 | 4.34±0.02 | 0.47 | 9.72 |
| 19 | 0.64±0.01 | 0.37±0.01 | 0.27 | 41.78[c] | 1.39±0.02 | 0.56±0.01 | 0.83 | 59.99[c] |
| Con[e] | 0.02±0.01 | 0.02±0.01 | 0 | 0 | 0.04±0.01 | 0.04±0.01 | 0 | 0 |

[a]Spot number refers to those shown in Fig. 3.4a.

[b]ASD ± SD = Average spot density ± Standard deviation.

[c]Student's t-test, $p \leq 0.05$.

[d]Protein spot was absent.

[e]Control group

### 3.6 Complementary DNA library screening and Phylogenetic tree

*An. campestris*-like cDNA libraries were constructed from the salivary glands of female dissected 3-5 day after emergence. Thirty randomly selected clones were sequenced from each cDNA library (Figure 3.5).

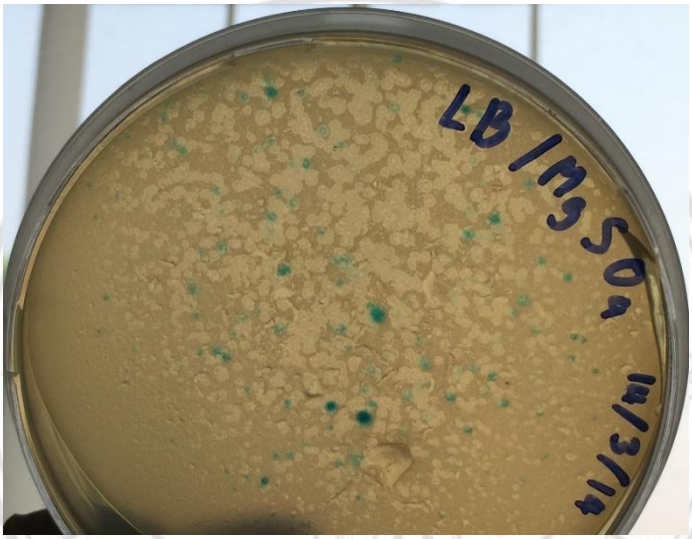


**Figure 3.5** *An. campestris*-like female salivary gland cDNA Library clone.

Sequence analysis showed that both A.cam-likeC16 and A.cam-likeC27 were full-length cDNA clones, but A.cam-likeC27 cDNA was longer than A.cam-likeC16 (Appendix section). ClustalW alignment of the A.cam-likeC16, A.cam-likeC27 and their similar sequences, are shown in the Appendix section. In addition, NJ Phylogenetic analysis of D7 proteins family showed several clades (Figure 3.6). BLASTx analysis revealed that the A.cam-likeC16 and A.cam-likeC27 matched the nucleotide sequence with 54 % and 53 % shared identity with a348 of *Anopheles anthropophagus*, respectively (Appendix section). Also, BLASTn analysis revealed that the A.cam-likeC16 and A.cam-likeC27 matched the nucleotide sequence with 66 % and 74 % shared identity with a mRNA for D7-related 1 protein of *An. stephensi,* respectively (Appendix section).

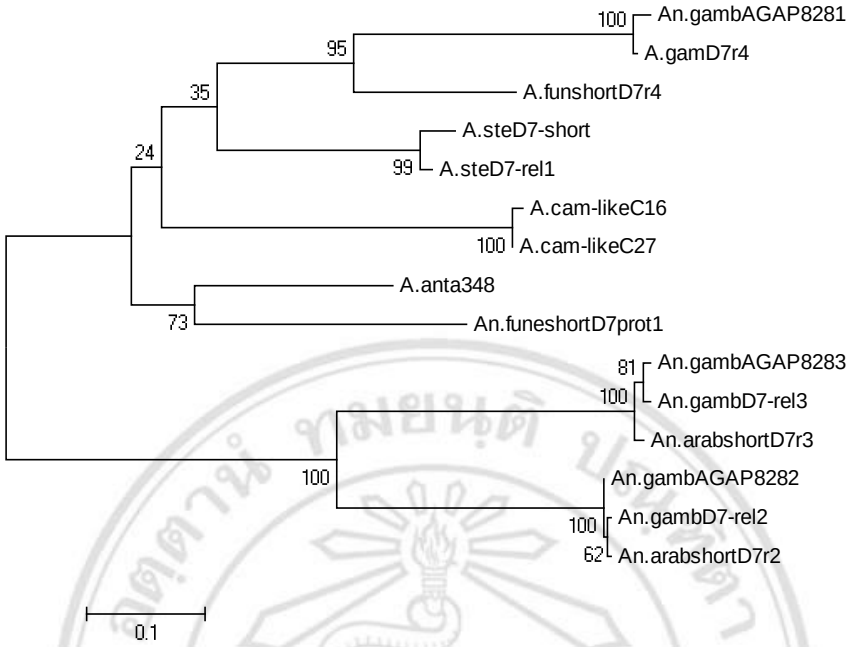


**Figure 3.6** NJ Phylogenetic analyses of the salivary proteins in the D7 family of *Anopheles* mosquitoes. The numbers on the branches represent the percentage of bootstrap support. The bar on the bottom represents 10% nucleotide sequence divergence.