# CHAPTER 3

# Results

***Anopheles nigerrimus***

## 3.1 Field Collections and Establishment of Isoline Colonies

Samples of fully engorged females of *An. nigerrimus* were collected from cow-baited traps in 4 provinces of Thailand and 1 location in Cambodia (Table 3.1, Figure 3.1). A total of 13 isolines were established and maintained in an insectary at Chiang Mai University, and they were used for studies on metaphase karyotype, cross-mating experiments and molecular analysis.

**Table 3.1** Locations in 5 provinces of Thailand and Cambodia, code of isolines, 4 karyotypic forms (A-D) of *An. nigerrimus* and their GenBank accession numbers

| Location (Geographical coordinate) | Code of isoline[a] | Karyotypic form | GenBank accession number | | | Reference |
|---|---|---|---|---|---|---|
| | | | ITS2 | COI | COII | |
| Thailand | | | | | | |
| Lampang (17° 53′ N, 99° 20′ E) | Lp1A[a] | A ($X_1$, $Y_1$) | AB778774 | AB778787 | AB778800 | This study |
| Ubon Ratchathani (15° 31′ N, 105° 35′ E) | Ur1A[a] | A ($X_2$, $Y_1$) | AB778775 | AB778788 | AB778801 | This study |
| | Ur7A | A ($X_2$, $Y_1$) | AB778776 | AB778789 | AB778802 | This study |
| | Ur20D[a] | D ($X_3$, $Y_4$) | AB778777 | AB778790 | AB778803 | This study |
| | Ur26A | A ($X_3$, $Y_1$) | AB778778 | AB778791 | AB778804 | This study |
| Nakhon Si Thammarat (08° 29′ N, 100° 0′ E) | Ns1B[a] | B ($X_2$, $Y_2$) | AB778779 | AB778792 | AB778805 | This study |
| | Ns2B | B ($X_3$, $Y_2$) | AB778780 | AB778793 | AB778806 | This study |
| | Ns3A[a] | A ($X_2$, $Y_1$) | AB778781 | AB778794 | AB778807 | This study |
| Songkhla (07° 13′ N, 100° 37′ E) | Sk2A[a] | A ($X_2$, $Y_1$) | AB778782 | AB778795 | AB778808 | This study |
| | Sk3A | A ($X_1$, $Y_1$) | AB778783 | AB778796 | AB778809 | This study |
| Cambodia | | | | | | |
| Ratanakiri (13° 44′ N, 107° 0′ E) | Rt2C[a] | C ($X_1$, $Y_3$) | AB778784 | AB778797 | AB778810 | This study |
| | Rt3C | C ($X_1$, $Y_3$) | AB778785 | AB778798 | AB778811 | This study |
| | Rt4A[a] | A ($X_1$, $Y_1$) | AB778786 | AB778799 | AB778812 | This study |

**Table 3.1** (continued)

| Location (Geographical coordinate) | Code of isoline[a] | Karyotypic form | GenBank accession number | | | Reference |
|---|---|---|---|---|---|---|
| | | | ITS2 | COI | COII | |
| Kalimantan, Indonesia | | | | | | |
| Hyrcanus Group | K13 | - | HM488261 | - | - | Paredes-Esquivel et al. 2011 |
| | K22 | - | HM488263 | - | - | Paredes-Esquivel et al. 2011 |
| | K26 | - | HM488267 | - | - | Paredes-Esquivel et al. 2011 |
| *An. belenrae* | - | - | EU789794 | - | - | Park et al. 2008a |
| *An. crawfordi* | Sk1B | B ($X_3$, $Y_2$) | AB779152 | AB779181 | AB779210 | Saeung et al. 2014 |
| *An. kleini* | - | - | EU789793 | - | - | Park et al. 2008a |
| *An. lesteri* | - | - | EU789791 | - | - | Park et al. 2008a |
| | ilG1 | - | - | AB733028 | AB733036 | Taai et al. 2013a |
| *An. nitidus* | Ur2D | D ($X_3$, $Y_4$) | AB777782 | AB777803 | AB777824 | This study |
| *An. paraliae* | Sk1B | B ($X_1$, $Y_2$) | AB733487 | AB733503 | AB733519 | Taai et al. 2013b |
| *An. peditaeniatus* | RbB | B ($X_3$, $Y_2$) | AB539061 | AB539069 | AB539077 | Choochote 2011 |
| *An. pullus* | - | - | EU789792 | - | - | Park et al. 2008a |
| | - | - | - | AY444348 | AY444347 | Park et al. 2003 |
| *An. sinensis* | i2ACM | A (X, $Y_1$) | AY130473 | - | - | Min et al. 2002 |
| | - | - | - | AY444351 | - | Park et al. 2003 |
| | i1BKR | B (X, $Y_2$) | - | - | AY130464 | Min et al. 2002 |

*a*: used in cross-mating experiments

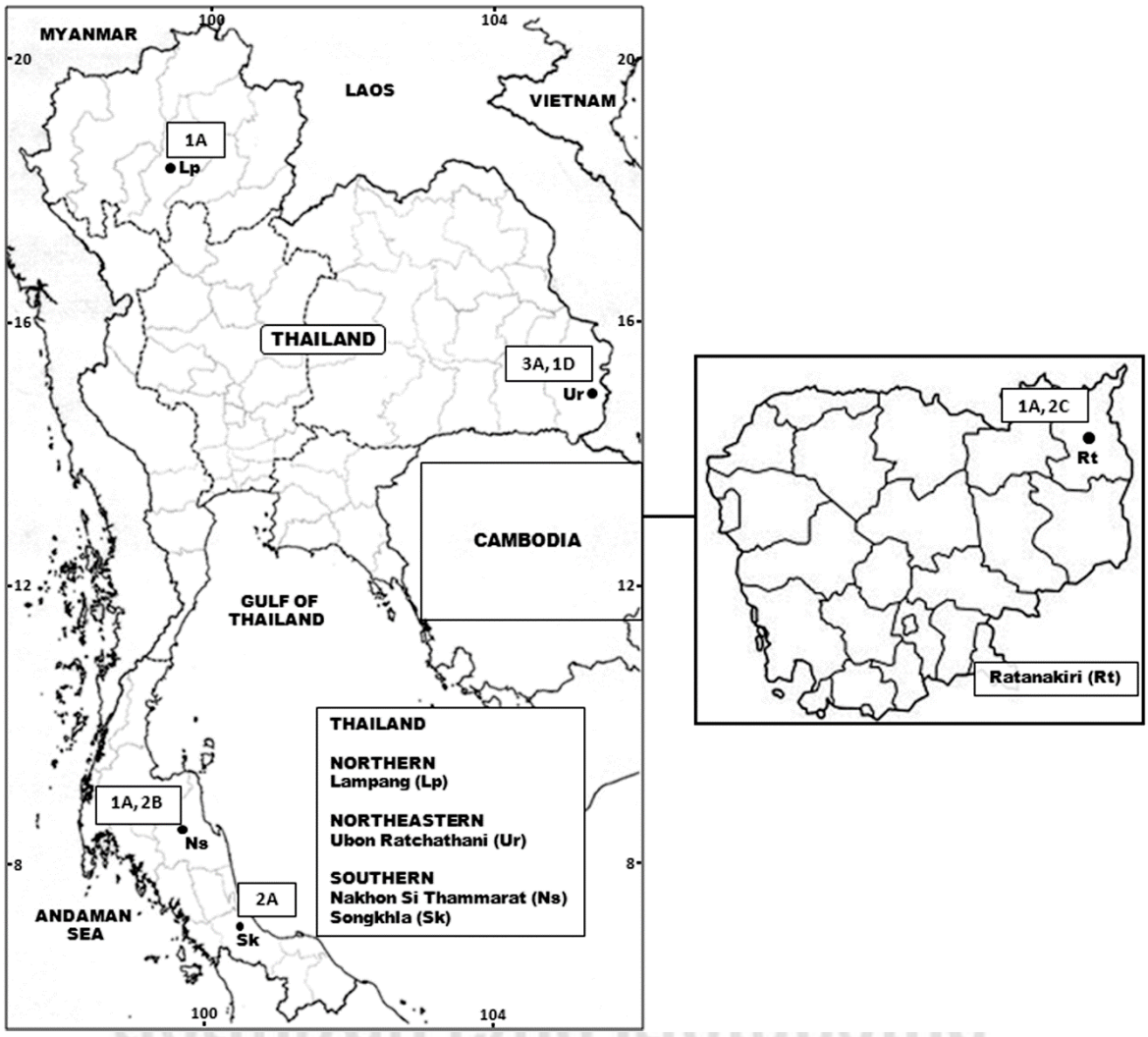


**Figure 3.1** Map of Thailand and Cambodia showing 5 provinces where samples of *An. nigerrimus* were collected and the numbers of isolines of the 4 karyotypic forms (A-D) were detected

### 3.2 Metaphase Karyotype of *Anopheles nigerrimus*

Cytogenetic observation of $F_1$- and/or $F_2$- progenies of the 13 isolines which were represented in 5 provinces from Thailand and Cambodia, demonstrated that *An. nigerrimus* has the typical chromosome number of 2n = 6, consisting of two pairs of autosomes (submetacentric and metacentric) and one pair of heteromorphic sex chromosomes (XX in females and XY in males). Based on the additional of extra block(s) of heterochromatin present in the heterochromatic arm of the sex chromosomes, 3 types of X (submetacentric $X_1$, large submetacentric $X_2$ and small metacentric $X_3$) and 4 types of Y chromosomes (large subtelocentric $Y_1$, submetacentric $Y_2$, small telocentric $Y_3$ and small subtelocentric $Y_4$) were obtained in this investigation (Table 3.1 and Figure 3.2). The $X_3$, $Y_3$ and $Y_4$ chromosomes were discovered in the present study. Based on the figures of $X_3$ and $Y_3$ chromosomes, they were probably represented the ancestral forms of X and Y chromosomes, respectively (Figure 3.2). There were 4 karyotypic forms on the basis of X and Y chromosome configurations, which were designated as Forms A ($X_1$, $X_2$, $X_3$, $Y_1$), B ($X_2$, $X_3$, $Y_2$), C ($X_1$, $Y_3$) and D ($X_3$, $Y_4$). Forms C and D were new karyotypic forms discovered in the present investigation. The number of isolines of these karyotypic forms occurring in different locations of Thailand and Cambodia are demonstrated in Table 3.1 and Figure 3.2. Form A appeared to be common in both Thailand and Cambodia. Form B and D were found rather specific in southern and northeastern Thailand, respectively, whereas Form C was confined somewhat to Cambodia.

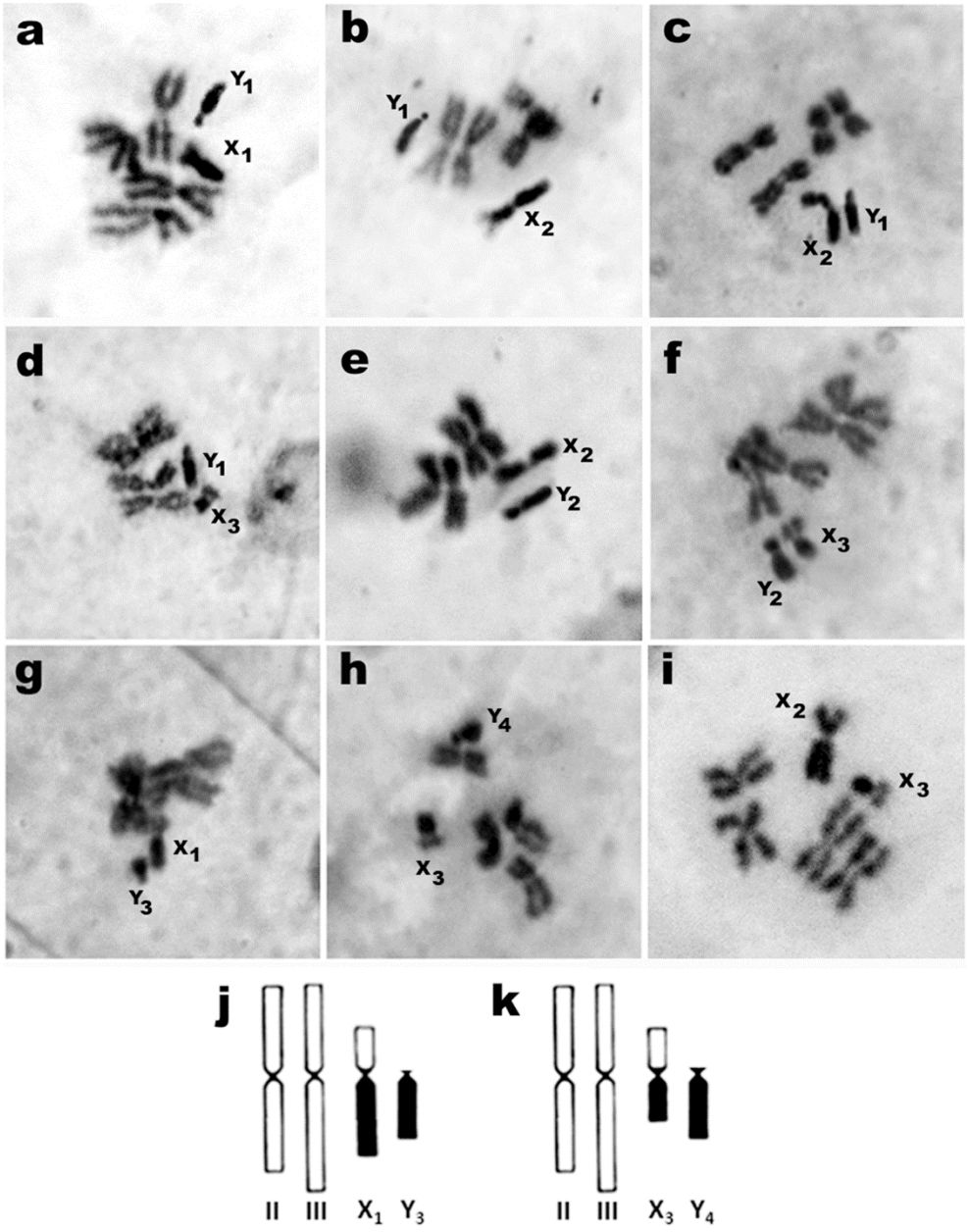




**Figure 3.2** Metaphase karyotypic forms of *An. nigerrimus*. (a) Form A ($X_1$, $Y_1$: Lampang); (b) Form A ($X_2$, $Y_1$: Ubon Ratchathani); (c) Form A ($X_2$, $Y_1$: Songkhla); (d) Form A ($X_3$, $Y_1$: Ubon Ratchathani); (e) Form B ($X_2$, $Y_2$: Nakhon Si Thammarat); (f) Form B ($X_3$, $Y_2$: Nakhon Si Thammarat); (g) Form C ($X_1$, $Y_3$: Ratanakiri); (h) Form D ($X_3$, $Y_4$: Ubon Ratchathani); (i) Form A (heterozygous $X_2$, $X_3$: Ubon Ratchathani); diagrams of representative metaphase karyotype of Form C (j) and Form D (k)

### 3.3 Cross-mating Experiments

Details of hatchability, pupation, emergence and adult sex ratio of parental, reciprocal and $F_1$-hybrid crosses among the 8 isolines of *An. nigerrimus* representing Forms A-D were 79.06-94.05%, 91.14-100%, 94.64-100%, and 0.81-1.12; 71.97-92.01%, 85.07-100%, 87.23-100%, and 0.89-1.14; 67.86-92.97%, 87.00-100%, 89.82-98.17%, and 0.71-1.27, respectively (Table 3.2). All yielded gave viable progenies through the $F_2$-generations. No evidence of genetic incompatibility and/or post-mating reproductive isolation was observed among these crosses. The salivary gland polytene chromosomes of the $4^{th}$ instar larvae of $F_1$-hybrids from all crosses showed complete synapsis without inversion loops in all chromosome arms (Figure 3.3).

**Table 3.2** Crossing experiments among the 8 isolines of *An. nigerrimus*

| Crosses (Female x Male) | Total eggs (number)[a] | Embryonation rate[b] | Hatched n (%) | Pupation n (%) | Emergence n (%) | Total emergence n (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Female | Male |
| **Parental cross** | | | | | | | |
| Lp1A x Lp1A | 320 (158, 162) | 87 | 253 (79.06) | 235 (92.89) | 235 (100.00) | 106 (45.11) | 129 (54.89) |
| Ur1A x Ur1A | 366 (194, 172) | 87 | 304 (83.06) | 304 (100.00) | 301 (99.01) | 158 (52.49) | 143 (47.51) |
| Sk2A x Sk2A | 399 (190, 209) | 85 | 331 (82.96) | 321 (96.98) | 314 (97.82) | 165 (52.55) | 149 (47.45) |
| Ns2A x Ns2A | 318 (161, 157) | 94 | 296 (93.08) | 296 (100.00) | 296 (100.00) | 145 (48.99) | 151 (51.01) |
| Rt4A x R43A | 332 (206, 126) | 94 | 295 (88.86) | 280 (94.92) | 265 (94.64) | 130 (49.06) | 135 (50.94) |
| Ns1B x Ns1B | 394 (194, 200) | 91 | 347 (88.07) | 333 (95.97) | 330 (99.10) | 174 (52.73) | 156 (47.27) |
| Rt2C x Rt2C | 359 (158, 201) | 94 | 316 (88.02) | 288 (91.14) | 278 (96.53) | 139 (50.00) | 139 (50.00) |
| Ur20D x Ur20D | 336 (210, 126) | 94 | 316 (94.05) | 316 (100.00) | 303 (95.89) | 136 (44.88) | 167 (55.12) |
| **Reciprocal cross** | | | | | | | |
| Lp1A x Ur1A | 353 (194, 159) | 96 | 300 (84.99) | 264 (88.00) | 255 (96.59) | 135 (52.94) | 120 (47.06) |
| Ur1A x Lp1A | 349 (158, 191) | 90 | 297 (85.10) | 267 (89.90) | 261 (97.75) | 130 (49.81) | 131 (50.19) |
| Lp1A x Sk2A | 364 (197, 167) | 88 | 309 (84.89) | 284 (91.91) | 262 (92.25) | 132 (50.38) | 130 (49.62) |
| Sk2A x Lp1A | 381 (194, 187) | 92 | 335 (87.93) | 328 (97.91) | 318 (96.95) | 164 (51.57) | 154 (48.43) |
| Lp1A x Ns3A | 350 (150, 200) | 89 | 298 (85.14) | 292 (97.99) | 283 (96.92) | 149 (52.65) | 134 (47.35) |
| Ns3A x Lp1A | 329 (157, 172) | 92 | 286 (86.93) | 280 (97.90) | 260 (92.86) | 137 (52.69) | 123 (47.31) |
| Lp1A x Rt4A | 311 (150, 161) | 94 | 274 (88.10) | 274 (100.00) | 263 (95.99) | 134 (50.95) | 129 (49.05) |
| Rt4A x Lp1A | 275 (157, 118) | 90 | 228 (82.91) | 219 (96.05) | 214 (97.72) | 114 (53.27) | 100 (46.73) |
| Lp1A x Ns1B | 356 (186, 170) | 94 | 317 (89.04) | 307 (96.85) | 301 (98.05) | 142 (47.18) | 159 (52.82) |

**Table 3.2** (continued)

| Crosses (Female x Male) | Total eggs (number)[a] | Embryonation rate[b] | Hatched n (%) | Pupation n (%) | Emergence n (%) | Total emergence n (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Female | Male |
| Ns1B x Lp1A | 291 (127, 164) | 86 | 221 (75.95) | 188 (85.07) | 164 (87.23) | 87 (53.05) | 77 (46.95) |
| Lp1A x Rt2C | 378 (269, 109) | 93 | 333 (88.10) | 333 (100.00) | 313 (94.00) | 147 (46.96) | 166 (53.04) |
| Rt2C x Lp1A | 346 (186, 160) | 81 | 249 (71.97) | 232 (93.17) | 222 (95.69) | 105 (47.30) | 117 (52.70) |
| Lp1A x Ur20D | 363 (128, 235) | 93 | 334 (92.01) | 331 (99.10) | 298 (90.03) | 156 (52.35) | 142 (47.65) |
| Ur20D x Lp1A | 346 (149, 197) | 92 | 311 (89.88) | 299 (96.14) | 299 (100.00) | 147 (49.16) | 152 (50.84) |
| **$F_1$-hybrid cross** | | | | | | | |
| (Lp1A x Ur1A)$F_1$ x (Lp1A x Ur1A)$F_1$ | 342 (112, 230) | 92 | 308 (90.06) | 308 (100.00) | 286 (92.86) | 160 (55.94) | 126 (44.06) |
| (Ur1A x Lp1A)$F_1$ x (Ur1A x Lp1A)$F_1$ | 287 (177, 110) | 92 | 258 (89.90) | 240 (93.02) | 226 (94.17) | 94 (41.59) | 132 (58.41) |
| (Lp1A x Sk2A)$F_1$ x (Lp1A x Sk2A)$F_1$ | 313 (172, 141) | 94 | 291 (92.97) | 274 (94.16) | 260 (94.89) | 115 (44.23) | 145 (55.77) |
| (Sk2A x Lp1A)$F_1$ x (Sk2A x Lp1A)$F_1$ | 329 (159, 170) | 79 | 257 (78.12) | 226 (87.94) | 203 (89.82) | 99 (48.77) | 104 (51.23) |
| (Lp1A x Ns3A)$F_1$ x (Lp1A x Ns3A)$F_1$ | 346 (186, 160) | 81 | 249 (71.71) | 232 (93.17) | 222 (95.69) | 105 (47.30) | 117 (52.70) |
| (Ns3A x Lp1A)$F_1$ x (Ns3A x Lp1A)$F_1$ | 319 (162, 157) | 90 | 271 (84.95) | 255 (94.10) | 247 (96.86) | 130 (52.63) | 117 (47.37) |
| (Lp1A x Rt4A)$F_1$ x (Lp1A x Rt4A)$F_1$ | 338 (125, 213) | 88 | 277 (81.95) | 241 (87.00) | 235 (97.51) | 116 (49.36) | 119 (50.64) |
| (Rt4A x Lp1A)$F_1$ x (Rt4A x Lp1A)$F_1$ | 345 (189, 156) | 87 | 293 (84.93) | 255 (87.03) | 243 (95.29) | 126 (51.85) | 117 (48.15) |
| (Lp1A x Ns1B)$F_1$ x (Lp1A x Ns1B)$F_1$ | 284 (167, 117) | 87 | 233 (82.04) | 226 (97.00) | 221 (97.79) | 112 (50.68) | 109 (49.32) |

**Table 3.2** (continued)

| Crosses (Female x Male) | Total eggs (number)[a] | Embryonation rate[b] | Hatched n (%) | Pupation n (%) | Emergence n (%) | Total emergence n (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Female | Male |
| (Ns1B x Lp1A)$F_1$ x (Ns1B x Lp1A)$F_1$ | 336 (177, 159) | 82 | 228 (67.86) | 219 (96.05) | 215 (98.17) | 94 (43.72) | 121 (56.28) |
| (Lp1A x Rt2C)$F_1$ x (Lp1A x Rt2C)$F_1$ | 295 (134, 161) | 75 | 215 (72.88) | 213 (99.07) | 207 (97.18) | 100 (48.31) | 107 (51.69) |
| (Rt2C x Lp1A)$F_1$ x (Rt2C x Lp1A)$F_1$ | 339 (182, 157) | 88 | 271 (79.94) | 255 (94.10) | 250 (98.04) | 135 (54.00) | 115 (46.00) |
| (Lp1A x Ur20D)$F_1$ x (Lp1A x Ur20D)$F_1$ | 342 (112, 230) | 92 | 308 (90.06) | 308 (100.00) | 286 (92.86) | 160 (55.94) | 126 (44.06) |
| (Ur20D x Lp1A)$F_1$ x (Ur20D x Lp1A)$F_1$ | 287 (177, 110) | 92 | 258 (89.90) | 240 (93.02) | 226 (94.17) | 94 (41.59) | 132 (58.41) |

*a*: two selective egg-batches of inseminated females from each cross; *b*: dissection from 100 eggs; n = number

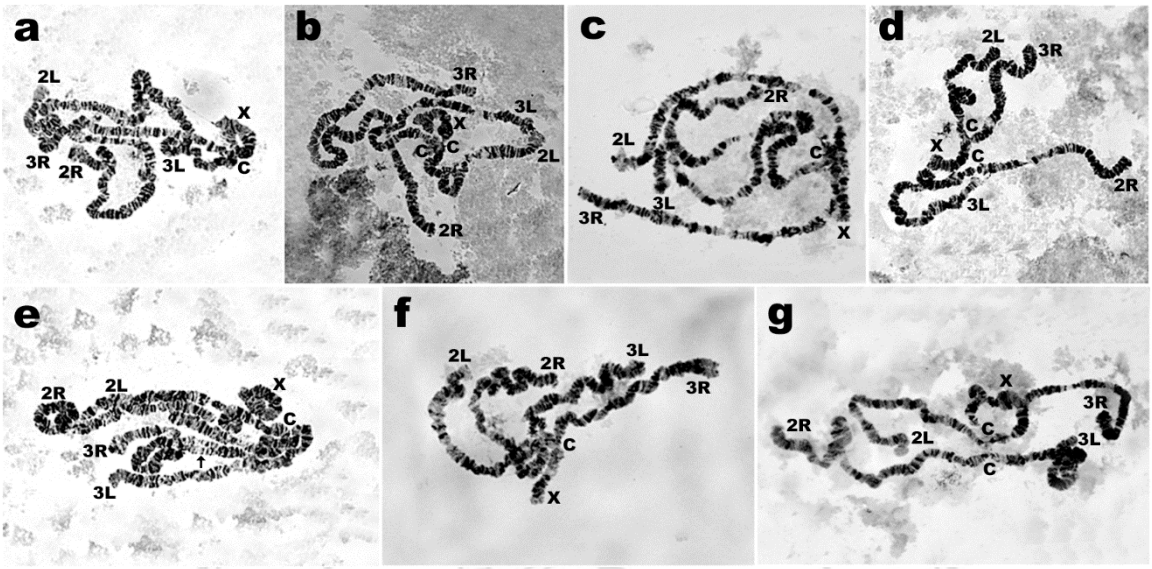


**Figure 3.3** Synapsis in all arms of salivary gland polytene chromosome of $F_1$-hybrid larvae of *An. nigerrimus*. (a) Lp1A female x Ur1A male; (b) Lp1A female x Sk2A male; (c) Lp1A female x Ns3A male; (d) Lp1A female x Rt4A male; (e) Lp1A female x Ns1B male, note: small gap of homosequential asynapsis was found on chromosome 3R (small arrow); (f ) Lp1A female x Rt2C male; (g) Lp1A female x Ur20D male

### 3.4 DNA Sequence and Phylogenetic Analysis

DNA sequences of the ITS2, COI and COII of the 13 isolines of *An. nigerrimus* Forms A-D from Thailand and Cambodia were analysed. They all showed the same lengths for ITS2 (508 bp), COI (658 bp) and COII (685 bp) sequences. Comparison of ITS2 sequences of 10 and 3 isolines from Thai and Cambodian *An. nigerrimus*, respectively, were performed. Among them, 8 were identical and the remaining 5 (Ns1B, Ns2B, Ns3A, Sk2A and Sk3A) from the southern region of Thailand shared the same nucleotide sequences, but they differed from the other 8 by only 2 nucleotide substitutions (A↔G at position 70, and T↔G at position 481). The evolutionary relationships were constructed among the 4 karyotypic forms using neighbor-joining and Bayesian trees. Both phylogenetic methods showed similar tree topologies, thus, only the Bayesian tree was shown for all regions (Figures 3.4 and 3.5). The average genetic distances within and between the 4 karyotypic forms exhibited no significant difference in three DNA regions (0.002-0.007) of both Thai and Cambodian populations. Therefore, the 13 isolines were placed within a single species, namely *An. nigerrimus*. Additionally, three ITS2 retrieved sequences (428 bp) from GenBank, previously identified as the Hyrcanus Group by Paredes-Esquivel et al. (2011), were grouped together with 4 karyotypic forms of *An. nigerrimus* (average genetic distances = 0.003). These ITS2 sequences were nearly identical to our sequences, and only 2 nucleotide substitutions were found among them. In support, the phylogenetic trees for ITS2, COI and COII of these isolines, representing Form A-D, were clearly different from other species of the Hyrcanus Group with strongly supported bootstrap values (Figures 3.4 and 3.5).

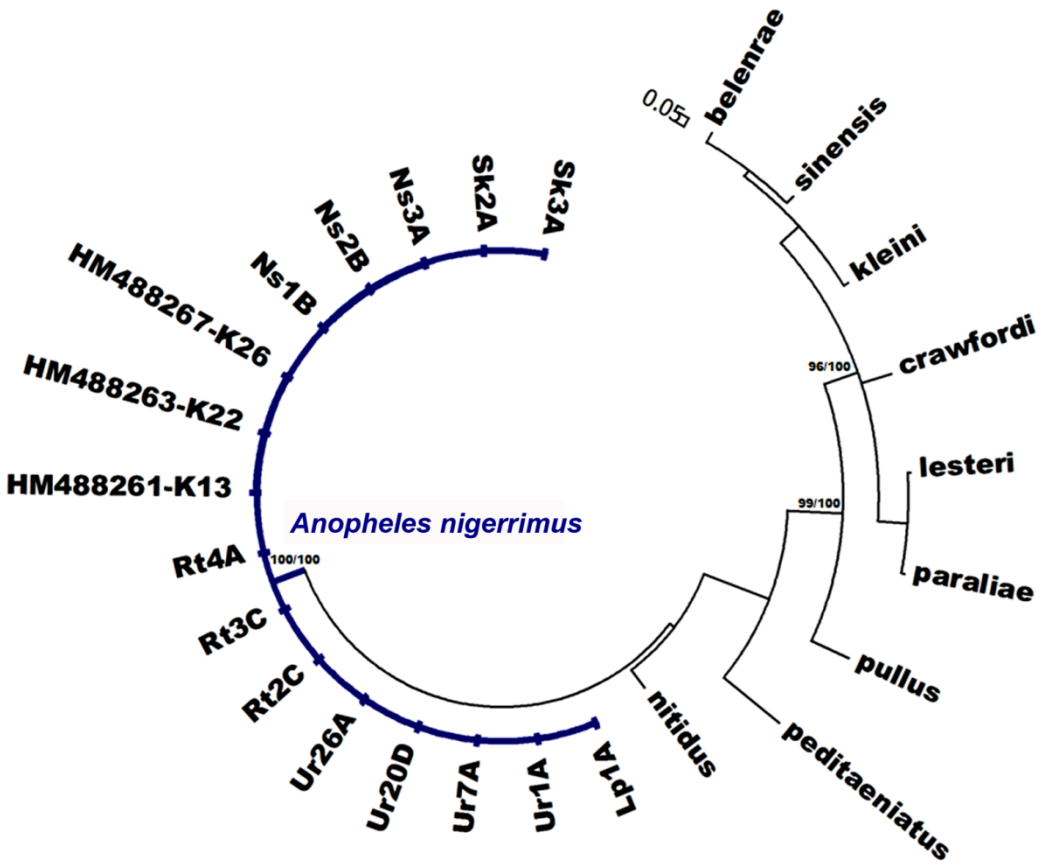


**Figure 3.4** Bayesian phylogenetic relationships among the 13 isolines of *An. nigerrimus* from Thailand and Cambodia based on ITS2 sequences compared with 9 species of the Hyrcanus Group and 3 specimens of the Hyrcanus Group from Kalimantan, Indonesia (Paredes-Esquivel et al. 2011). Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 70% both on bootstrap values and posterior probabilities are shown. Branch lengths are proportional to genetic distance (scale bar)

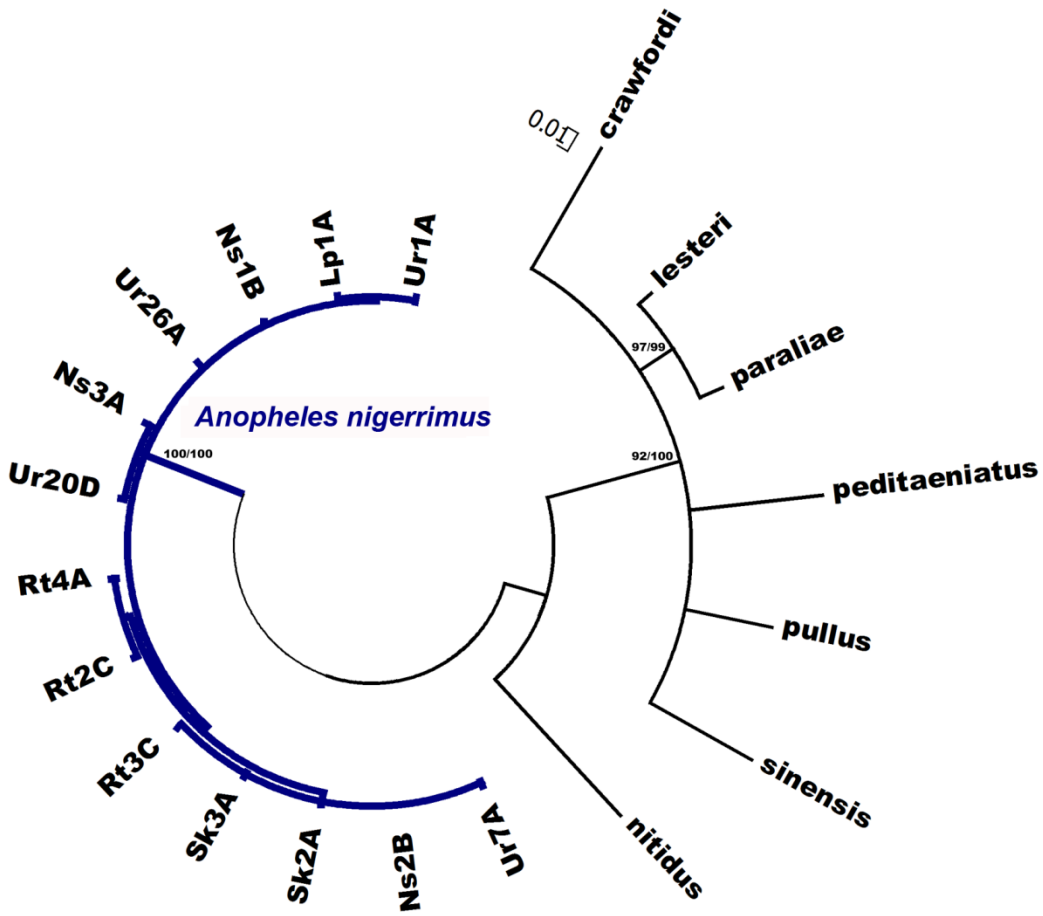


**Figure 3.5** Bayesian phylogenetic relationships among the 13 isolines of *An. nigerrimus* from Thailand and Cambodia, based on combined sequences of COI and COII, compared with 7 species of the Hyrcanus Group. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 70% both on bootstrap values and posterior probabilities are shown. Branch lengths are proportional to genetic distance (scale bar)

***Anopheles nitidus***

### 3.5 Field Collections and Establishment of Isoline Colonies

Samples of fully engorged females of *An. nitidus* were collected from cow-baited traps in 2 provinces of Thailand, i.e., Phang Nga and Ubon Ratchathani provinces (Table 3.3, Figure 3.6). A total of 21 isolines were established and maintained in an insectary at Chiang Mai University, and they were used for studies on metaphase karyotype, cross-mating experiments and molecular analysis.

**Table 3.3** Locations in 2 provinces of Thailand, code of isolines, 5 karyotypic forms (A-E) of *An. nitidus* and their GenBank accession numbers

| Location (Geographical coordinate) | Code of isoline[a] | Karyotypic form | Region | GenBank accession number | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | | ITS2 | COI | COII | |
| *An. nitidus* | | | | | | | |
| Ubon Ratchathani | Ur2D[a] | D ($X_3$, $Y_4$) | ITS2, COI, COII | AB777782 | AB777803 | AB777824 | This study |
| (15° 31′ N, 105° 35′ E) | Ur5E[a] | E ($X_2$, $Y_5$) | ITS2, COI, COII | AB777783 | AB777804 | AB777825 | This study |
| | Ur8E | E ($X_1$, $Y_5$) | ITS2, COI, COII | AB777784 | AB777805 | AB777826 | This study |
| | Ur11D | D ($X_3$, $Y_4$) | ITS2, COI, COII | AB777785 | AB777806 | AB777827 | This study |
| | Ur12D | D ($X_1$, $Y_4$) | ITS2, COI, COII | AB777786 | AB777807 | AB777828 | This study |
| | Ur15D | D ($X_3$, $Y_4$) | ITS2, COI, COII | AB777787 | AB777808 | AB777829 | This study |
| | Ur16E | E ($X_1$, $Y_5$) | ITS2, COI, COII | AB777788 | AB777809 | AB777830 | This study |
| | Ur19D | D ($X_1$, $Y_4$) | ITS2, COI, COII | AB777789 | AB777810 | AB777831 | This study |
| | Ur22E | E ($X_2$, $Y_5$) | ITS2, COI, COII | AB777790 | AB777811 | AB777832 | This study |
| | Ur23E | E ($X_3$, $Y_5$) | ITS2, COI, COII | AB777791 | AB777812 | AB777833 | This study |
| | Ur24D | D ($X_3$, $Y_4$) | ITS2, COI, COII | AB777792 | AB777813 | AB777834 | This study |
| | Ur25D | D ($X_1$, $Y_4$) | ITS2, COI, COII | AB777793 | AB777814 | AB777835 | This study |
| | Ur27D | D ($X_1$, $Y_4$) | ITS2, COI, COII | AB777794 | AB777815 | AB777836 | This study |
| | Ur28E | E ($X_3$, $Y_5$) | ITS2, COI, COII | AB777795 | AB777816 | AB777837 | This study |
| | Ur30E | E ($X_1$, $Y_5$) | ITS2, COI, COII | AB777796 | AB777817 | AB777838 | This study |
| | Ur31D | D ($X_3$, $Y_4$) | ITS2, COI, COII | AB777797 | AB777818 | AB777839 | This study |
| | Ur33E | E ($X_2$, $Y_5$) | ITS2, COI, COII | AB777798 | AB777819 | AB777840 | This study |
| | Ur34D | D ($X_3$, $Y_4$) | ITS2, COI, COII | AB777799 | AB777820 | AB777841 | This study |
| Phang Nga | Pg2A[a] | A ($X_1$, $Y_1$) | ITS2, COI, COII | AB777800 | AB777821 | AB777842 | This study |
| (08° 27′ N, 98° 31′ E) | Pg4C[a] | C ($X_2$, $Y_3$) | ITS2, COI, COII | AB777801 | AB777822 | AB777843 | This study |
| | Pg5B[a] | B ($X_1$, $Y_2$) | ITS2, COI, COII | AB777802 | AB777823 | AB777844 | This study |

**Table 3.3** (continued)

| Location (Geographical coordinate) | Code of isoline[a] | Karyotypic form | Region | GenBank accession number | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | | ITS2 | COI | COII | |
| Hyrcanus Group | TR2 | - | ITS2 | HM488273 | - | - | Paredes-Esquivel et al. 2011 |
| | TR3 | - | ITS2 | HM488272 | - | - | Paredes-Esquivel et al. 2011 |
| | TR6 | - | ITS2 | HM488268 | - | - | Paredes-Esquivel et al. 2011 |
| *An. belenrae* | - | - | ITS2 | EU789794 | - | - | Park et al. 2008a |
| *An. crawfordi* | Pg4A | A ($X_1$, $Y_1$) | ITS2, COI, COII | AB779142 | AB779171 | AB779200 | Saeung et al. 2014 |
| *An. kleini* | - | - | ITS2 | EU789793 | - | - | Park et al. 2008a |
| *An. lesteri* | - | - | ITS2 | EU789791 | - | - | Park et al. 2008a |
| | ilG1 | - | COI, COII | - | AB733028 | AB733036 | Taai et al. 2013a |
| *An. paraliae* | Sk1B | B ($X_1$, $Y_2$) | ITS2, COI, COII | AB733487 | AB733503 | AB733519 | Taai et al. 2013b |
| *An. peditaeniatus* | RbB | B ($X_3$, $Y_2$) | ITS2, COI, COII | AB539061 | AB539069 | AB539077 | Choochote 2011 |
| *An. pullus* | - | - | ITS2 | EU789792 | - | - | Park et al. 2008a |
| | | | COI, COII | - | AY444348 | AY444347 | Park et al. 2003 |
| *An. sinensis* | i2ACM | A (X, $Y_1$) | ITS2 | AY130473 | - | - | Min et al. 2002 |
| | - | - | COI | - | AY444351 | - | Park et al. 2003 |
| | i1BKR | B (X, $Y_2$) | COII | - | - | AY130464 | Min et al. 2002 |

*a*: used in cross-mating experiments

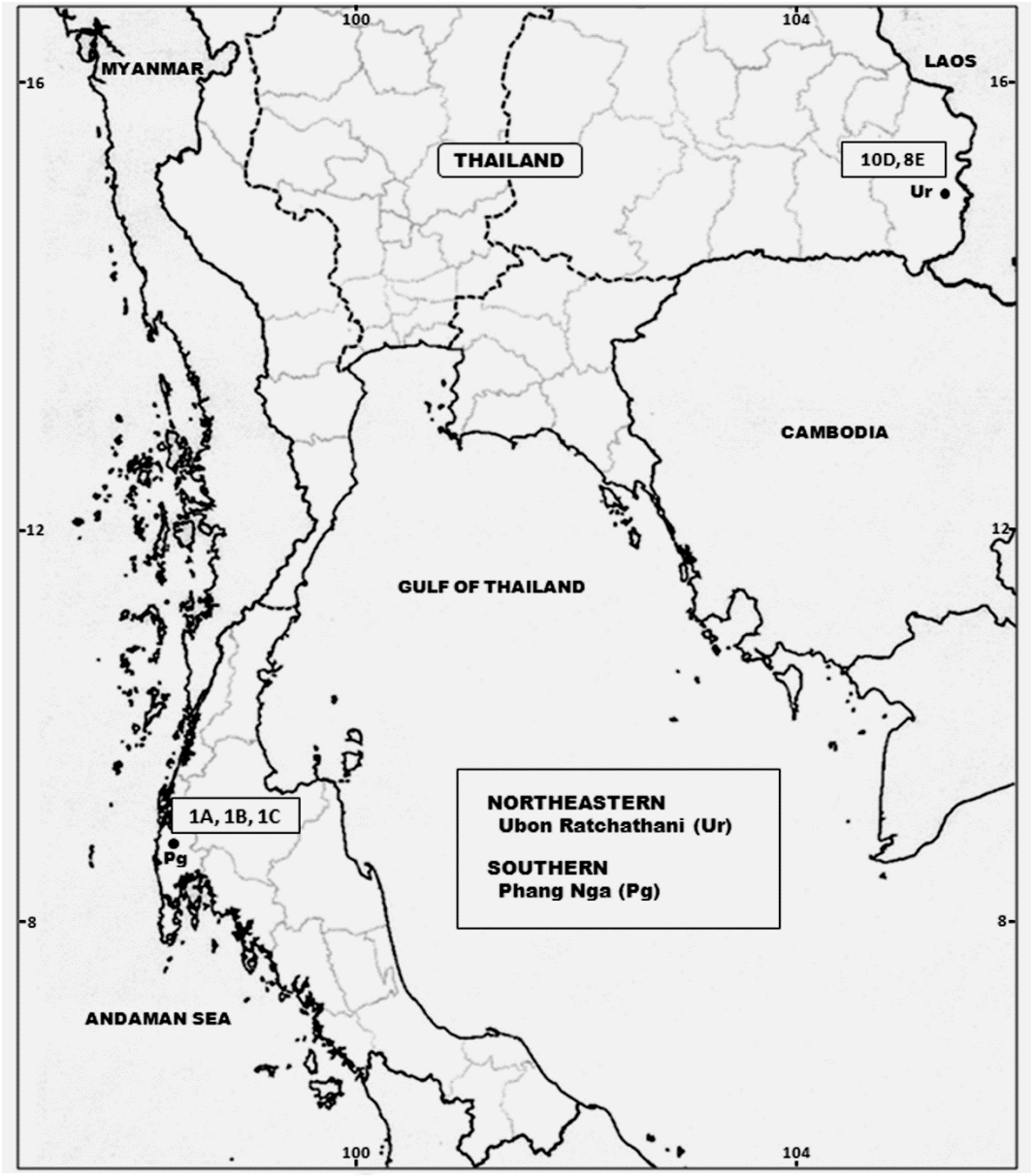


**Figure 3.6** Map of Thailand showing two provinces where specimens of *An. nitidus* were collected and the number of isolines of the five karyotypic forms (A–E) detected in each location

### 3.6 Metaphase Karyotype of *Anopheles nitidus*

Cytogenetic observations of $F_1$ progenies of the 21 isolines of *An. nitidus* which were represented in 2 provinces in Thailand, i.e., Phang Nga and Ubon Ratchathani provinces revealed different types of sex chromosomes due to the addition of extra block(s) of heterochromatin. There were 3 types of X (small metacentric $X_1$, submetacentric $X_2$ and large submetacentric $X_3$) and 5 types of Y chromosomes (small telocentric $Y_1$, small subtelocentric $Y_2$, large subtelocentric $Y_3$, submetacentric $Y_4$ and small metacentric $Y_5$) (Figures 3.7 and 3.8). The $X_1$ chromosome has a small metacentric with one arm euchromatic, and the opposite one totally heterochromatic. The $X_2$ chromosome is different from the $X_1$ chromosome in having an extra block of heterochromatin in the heterochromatic arm, making it a long arm of submetacentric. The $X_3$ chromosome has a large submetacentric that was slightly different from the $X_2$ chromosome in having an extra block of heterochromatin at the distal end of the long heterochromatic arm. A good comparison of the size and morphology between $X_2$ and $X_3$ chromosomes could be made easily in heterozygous females (Figure 3.7I). Similar to the situation in the X chromosome, the Y chromosome also exhibited extensive variation in size and morphology, due to differing amounts and distribution of heterochromatic block. Thus, the $Y_1$ chromosome is an apparently small telocentric, which represents the ancestral form (Figure 3.7A). The $Y_2$ chromosome has a small subtelocentric or acrocentric that slightly differs from the $Y_1$ chromosome, which has a very small portion of the short arm present (Figure 3.7B). Chromosome $Y_3$ has a large subtelocentric that obviously differs from the $Y_2$ chromosome in having an extra block of heterochromatin at the distal end of the long heterochromatic arm (Figure 3.7C). The $Y_4$ chromosome is clearly submetacentric, with the short arm approximately 1/3 the length of the long arm (Figure 3.7D and E). It appears to have derived from the $Y_3$ chromosome by means of adding an extra block of heterochromatin onto the short arm, and transferring it to a submetacentric. Chromosome $Y_5$ had a small metacentric, which was quite different from chromosomes $Y_1$, $Y_2$, $Y_3$ and $Y_4$ by having an equal heterochromatic block on each arm (Figure 3.7F and G). Based on uniquely different characteristics of Y chromosome from each isoline colony, they were designated as Form A ($X_1$, $Y_1$), Form B ($X_1$, $Y_2$), Form C ($X_2$, $Y_3$), Form D ($X_1$, $X_3$, $Y_4$) and Form E

($X_1$, $X_2$, $X_3$, $Y_5$). Forms A, B and C were found in Phang Nga province, and Forms D and E were obtained in Ubon Ratchathani province.

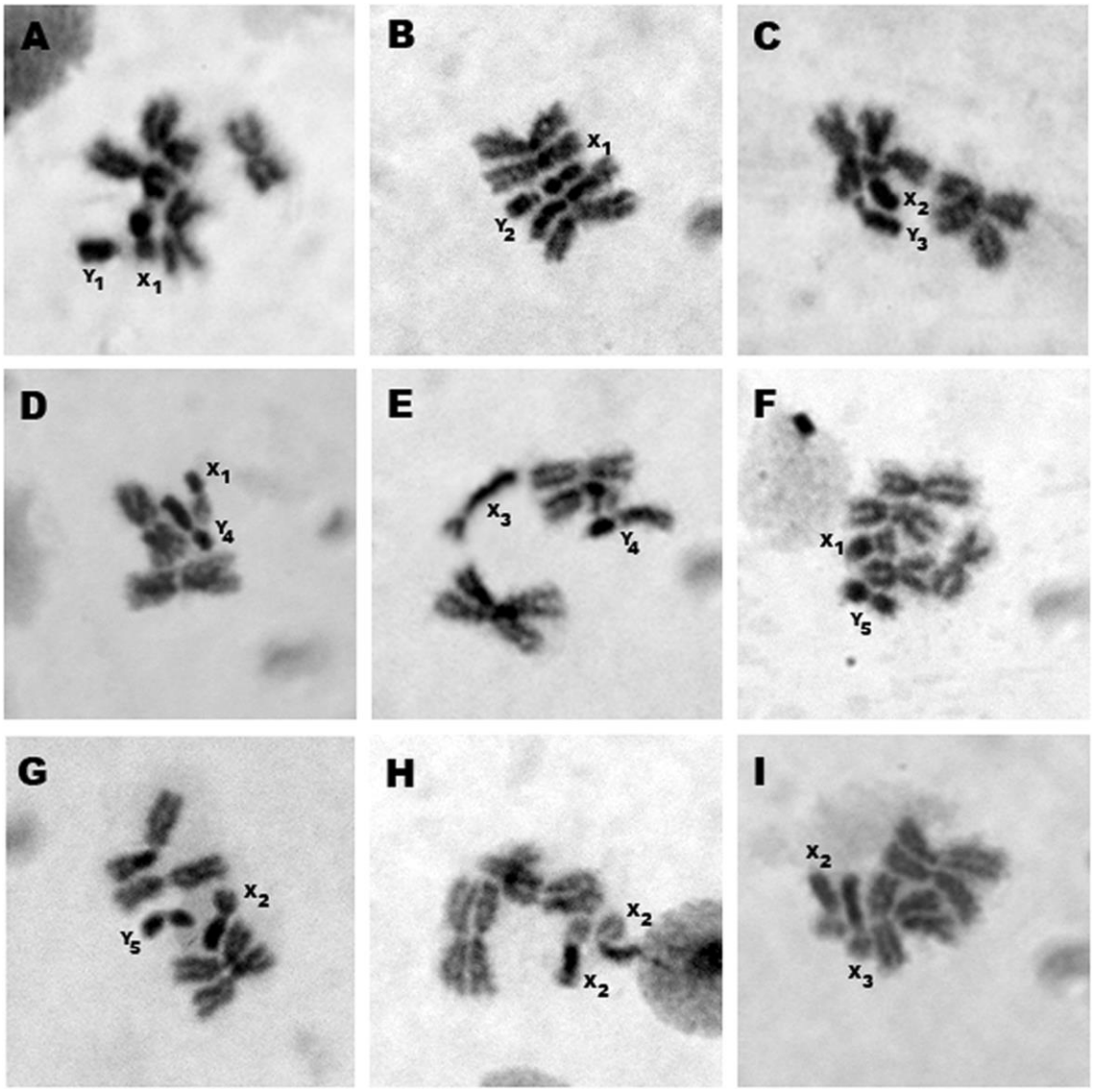


**Figure 3.7** Metaphase karyotypic forms of *An. nitidus*. Phang Nga province (A-C) (A) Form A ($X_1$, $Y_1$), (B) Form B ($X_1$, $Y_2$), (C) Form C ($X_2$, $Y_3$); Ubon Ratchathani province (D-I) (D) Form D ($X_1$, $Y_4$), (E) Form D ($X_3$, $Y_4$), (F) Form E ($X_1$, $Y_5$), (G) Form E ($X_2$, $Y_5$), (H) Form E (homozygous $X_2$, $X_2$), (I) Form E (heterozygous $X_2$, $X_3$)

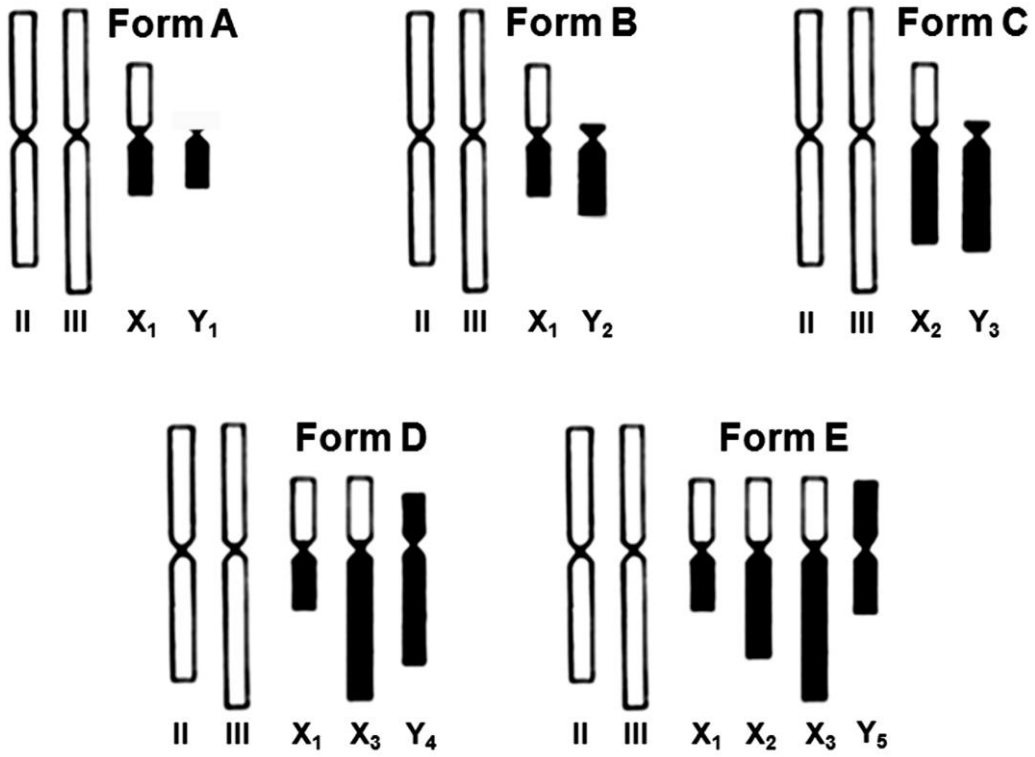


**Figure 3.8** Diagrams of representative metaphase karyotypes of Forms A, B, C, D and E of *An. nitidus*



### 3.7 Cross-mating Experiments

Details of hatchability, pupation, emergence and adult sex-ratio of parental, reciprocal and $F_1$-hybrid crosses among the 5 isolines of *An. nitidus* Forms A, B, C, D and E from Thailand are shown in Table 3.4. All crosses yielded viable progenies through $F_2$ generations. No evidence of genetic incompatibility and/or post-mating reproductive isolation was observed among these crosses. The salivary gland polytene chromosomes of the 4$^{th}$ stage larvae from all crosses showed synapsis without any inversion loops along the whole length of all autosomes and the X chromosome (Figure 3.9).

**Table 3.4** Cross-mating experiments among the 5 isolines of *An. nitidus*

| Crosses (Female x Male) | Total eggs (number)[a] | Embryonation rate[b] | Hatched n (%) | Pupation n (%) | Emergence n (%) | Total emergence n (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Female | Male |
| **Parental cross** | | | | | | | |
| Pg2A x Pg2A | 244 (125, 119) | 88 | 210 (86.06) | 195 (92.86) | 195 (100.00) | 103 (52.82) | 92 (47.18) |
| Pg5B x Pg5B | 277 (130, 147) | 91 | 238 (85.92) | 226 (94.96) | 221 (97.79) | 107 (48.42) | 114 (51.58) |
| Pg4C x Pg4C | 283 (118, 165) | 84 | 218 (77.03 ) | 218 (100.00) | 211 (96.79) | 106 (50.24) | 105 (49.76) |
| Ur2D x Ur2D | 292 (109, 183) | 92 | 263 (90.07 ) | 258 (98.10) | 247 (95.74) | 131 (53.04) | 116 (46.96) |
| Ur5E x Ur5E | 301 (148, 153) | 88 | 256 (85.05) | 251 (98.05) | 221 (88.05) | 111 (50.23) | 110 (49.77) |
| **Reciprocal cross** | | | | | | | |
| Pg2A x Pg5B | 289 (147, 142) | 94 | 260 (89.97) | 257 (98.85) | 239 (93.00) | 117 (48.95) | 122 (51.05) |
| Pg5B x Pg2A | 298 (158, 140) | 90 | 220 (73.83) | 202 (91.82) | 198 (98.02) | 97 (48.99) | 101 (51.01) |
| Pg2A x Pg4C | 299 (131, 168) | 92 | 260 (86.96) | 231 (88.85) | 226 (97.84) | 112 (49.56) | 114 (50.44) |
| Pg4C x Pg2A | 313 (162, 151) | 80 | 225 (71.88) | 218 (96.89) | 209 (95.87) | 112 (53.59) | 97 (46.41) |
| Pg2A x Ur2D | 211 (103, 108) | 86 | 175 (82.94) | 159 (90.86) | 159 (100.00) | 64 (40.25) | 95 (59.75) |
| Ur2D x Pg2A | 224 (111, 113) | 91 | 202 (90.18) | 196 (97.03) | 171 (87.24) | 81 (47.37) | 90 (52.63) |
| Pg2A x Ur5E | 243 (118, 125) | 87 | 207 (85.19) | 207 (100.00) | 197 (95.17) | 100 (50.76) | 97 (49.24) |
| Ur5E x Pg2A | 264 (139, 125) | 91 | 235 (89.02) | 235 (100.00) | 204 (86.81) | 108 (52.94) | 96 (47.06) |

**Table 3.4** (continued)

| Crosses (Female x Male) | Total eggs (number)[a] | Embryonation rate[b] | Hatched n (%) | Pupation n (%) | Emergence n (%) | Total emergence n (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Female | Male |
| **$F_1$- hybrid cross** | | | | | | | |
| (Pg2A x Pg5B)$F_1$ x (Pg2A x Pg5B)$F_1$ | 308 (118, 190) | 85 | 246 (79.87) | 234 (95.12) | 229 (97.86) | 111 (48.47) | 118 (51.53) |
| (Pg5B x Pg2A)$F_1$ x (Pg5B x Pg2A)$F_1$ | 312 (186, 126) | 87 | 250 (80.13) | 235 (94.00) | 225 (95.74) | 110 (48.89) | 115 (51.11) |
| (Pg2A x Pg4C)$F_1$ x (Pg2A x Pg4C)$F_1$ | 308 (147, 161) | 92 | 271 (87.99) | 268 (98.89) | 257 (95.90) | 135 (52.53) | 122 (47.47) |
| (Pg4C x Pg2A)$F_1$ x (Pg4C x Pg2A)$F_1$ | 329 (194, 135) | 80 | 250 (75.99) | 230 (92.00) | 225 (97.83) | 115 (51.11) | 110 (48.89) |
| (Pg2A x Ur2D)$F_1$ x (Pg2A x Ur2D)$F_1$ | 347 (157, 190) | 90 | 295 (85.01) | 289 (97.97) | 265 (91.70) | 141 (53.21) | 124 (46.79) |
| (Ur2D x Pg2A)$F_1$ x (Ur2D x Pg2A)$F_1$ | 287 (125, 162) | 90 | 250 (87.11) | 222 (88.80) | 220 (99.10) | 112 (50.91) | 108 (49.09) |
| (Pg2A x Ur5E)$F_1$ x (Pg2A x Ur5E)$F_1$ | 350 (167, 183) | 88 | 280 (80.00) | 272 (97.14) | 266 (97.79) | 126 (47.37) | 140 (52.63) |
| (Ur5E x Pg2A)$F_1$ x (Ur5E x Pg2A)$F_1$ | 339 (194, 145) | 84 | 268 (79.06) | 263 (98.13) | 242 (92.02) | 124 (51.24) | 118 (48.76) |

*a*: two selective egg-batches of inseminated females from each cross; *b*: dissection from 100 eggs; n = number

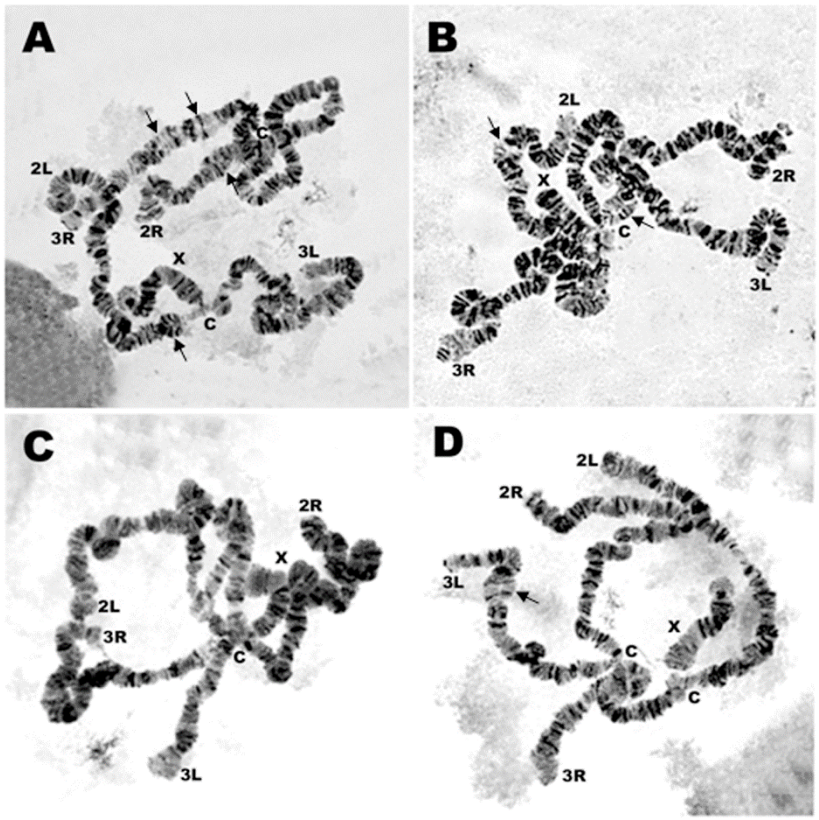


**Figure 3.9** Synapsis in all arms of salivary gland polytene chromosome of $F_1$-hybrids 4$^{th}$ larvae of *An. nitidus*. (A) Pg2A female x Pg5B male; (B) Pg2A female x Pg4C male; (C) Pg2A female x Ur2D male; (D) Pg2A female x Ur5E male. Note: small common gap of homosequential asynapsis (arrow) was found on chromosome 2L, 2R and 3R; 2L and 2R; and 3L from the crosses between Pg2A female x Pg5B male; Pg2A female x Pg4C male; and Pg2A female x Ur5E male, respectively

### 3.8 DNA Sequence and Phylogenetic Analysis

DNA sequences were determined and analyzed for the ITS2, COI and COII of the 21 isolines of *An. nitidus* Forms A, B, C, D and E. They showed various lengths of ITS2, at 480 bp in 18 isolines from Ubon Ratchathani province and 481 bp in 3 isolines from Phang Nga province. The *An. nitidus* from Ubon Ratchathani province differed from that in Phang Nga province by a deletion of T at position 421. They all showed the same length in COI (658 bp) and COII (685 bp). Neighbor-joining (NJ) and Bayesian trees were constructed to reveal the evolutionary relationship of the five karyotypic forms. Both phylogenetic methods showed similar tree topologies, thus, only the Bayesian tree is shown in Figures 3.10 and 3.11 The results showed that all sequences of *An. nitidus* Forms A, B, C, D and E were monophyletic in both trees, with high support (NJ = 99-100%, BPP = 100%). The average genetic distances within the five karyotypic forms (21 isolines) of *An. nitidus* were 0.002, 0.008 and 0.006 for ITS2, COI and COII sequences, respectively. Furthermore, all karyotypic forms of *An. nitidus* were well separated from other species members (*An. belenrae, An. crawfordi, An. kleini, An. lesteri, An. paraliae, An. peditaeniatus, An. pullus* and *An. sinensis*) of the Hyrcanus Group (Figures 3.10 and 3.11). The three published ITS2 sequences (GenBank accession numbers HM488268, HM488272 and HM488273, Table 3.3), which were identified previously as the Hyrcanus Group, also were placed within the same clade of *An. nitidus* (Figure 3.10).

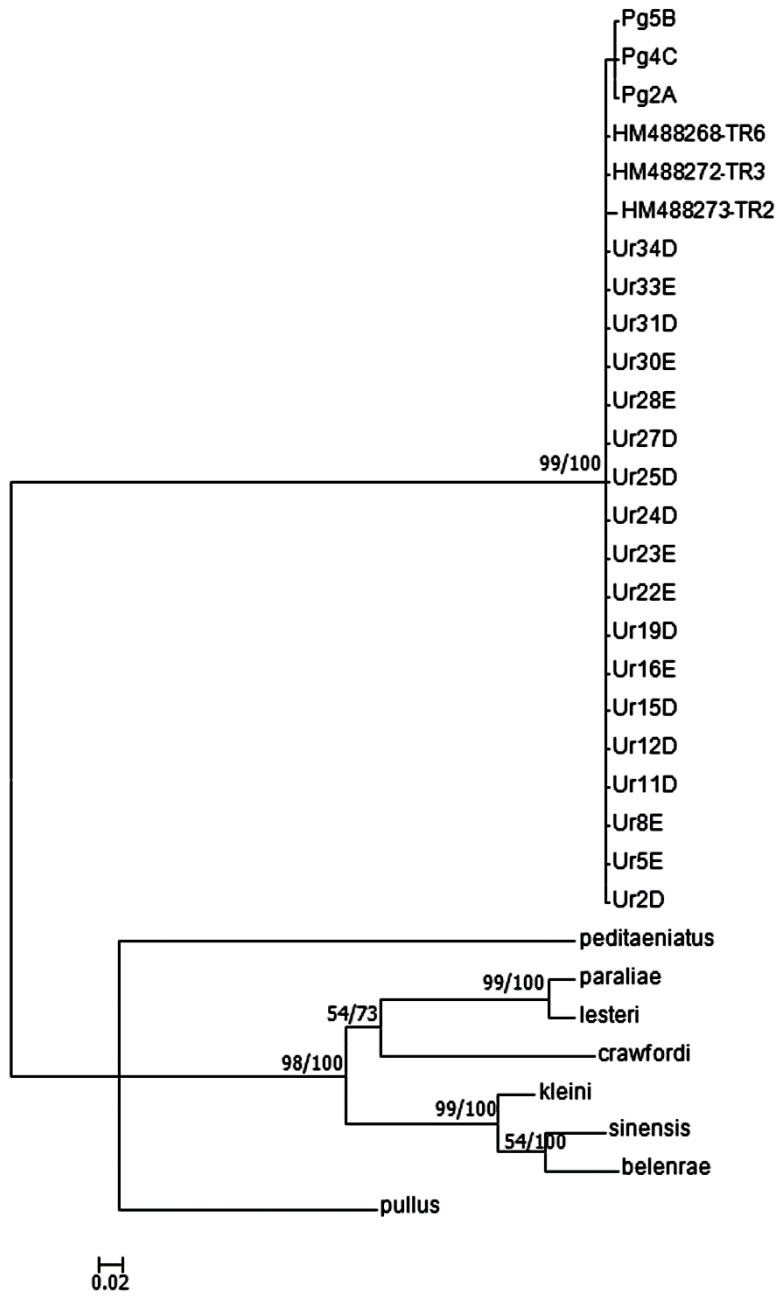


**Figure 3.10** Phylogenetic relationships of the 5 karyotypic forms of *An. nitidus* using Bayesian analysis based on ITS2 sequences compared with 3 specimens from Trat Province (Paredes-Esquivel et al. 2011) and 8 species of the Hyrcanus Group. Codes for the specimens are shown in Table 3.3. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 50% are shown. Branch lengths are proportional to genetic distance (scale bar)

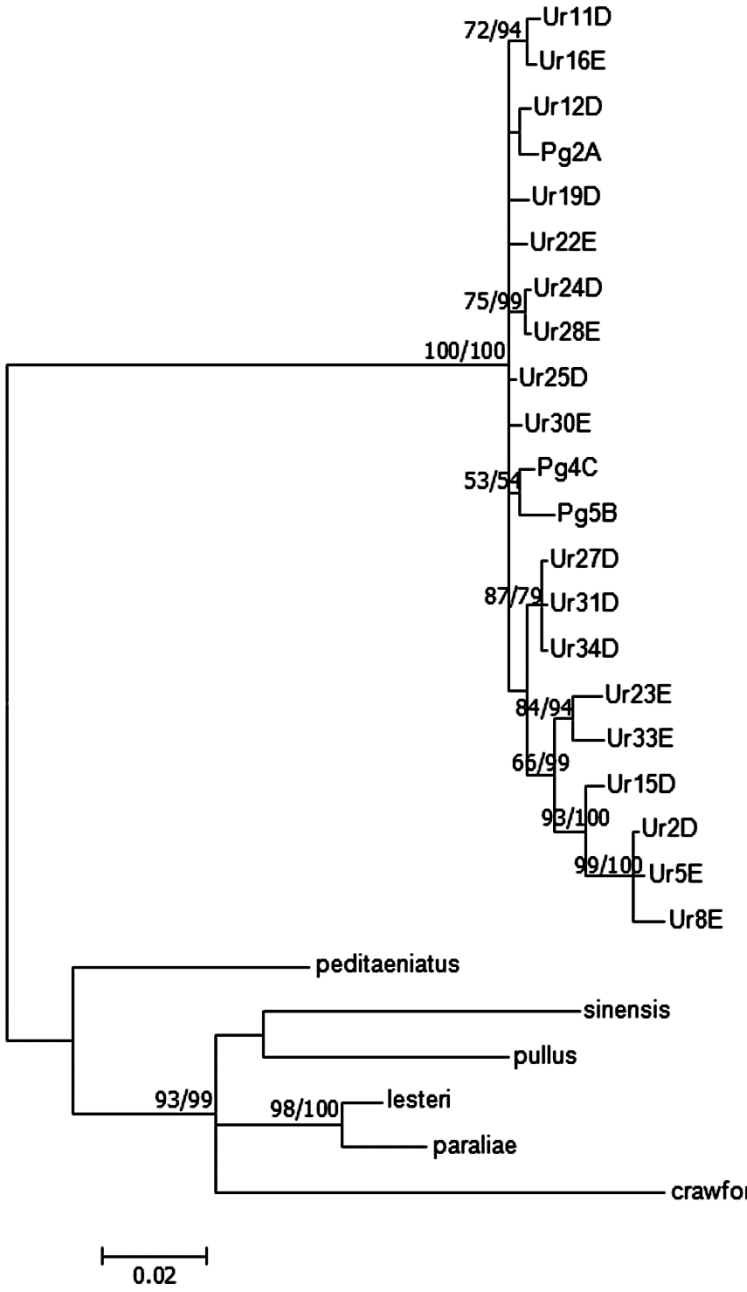




**Figure 3.11** Phylogenetic relationships among the 5 karyotypic forms of *An. nitidus* using Bayesian analysis based on combined COI and COII sequences compared with 6 species of the Hyrcanus Group. Codes for the specimens are shown in Table 3.3. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 50% are shown. Branch lengths are proportional to genetic distance (scale bar)