สัณฐานวิทยา เซลล์พันธุศาสตร์ และดีเอ็นเอบาร์โค้ดของริ้นน้ำจืด (Diptera)ในประเทศไทย
Morphology, Cytogenetics and DNA barcode of the Chironomidae (Diptera) in Thailand

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บทคัดย่อ

ริ้นน้ำจืดเป็นแมลงในวงศ์ Chironomidae มีความสำคัญต่อระบบนิเวศน้ำจืด อย่างไรก็ตามข้อมูลอนุกรมวิธานของริ้นน้ำจืดในประเทศไทยยังมีการศึกษาไม่มาก ในการศึกษานี้ใช้ลักษณะในการศึกษาอนุกรมวิธานหลายระดับเพื่อใช้ในการจำแนกชนิดของริ้นน้ำจืด 3 สปีชีส์ได้แก่ *Chironomus striatipennis*, *C. javanus* และ *Kiefferulus tainanus* โดยเก็บตัวอย่างริ้นน้ำจืดจากแหล่งน้ำในจังหวัดมหาสารคามและจังหวัดร้อยเอ็ด ผลการศึกษาพบว่าลักษณะสัณฐานวิทยาของริ้นน้ำจืดทั้ง 3 สปีชีส์สอดคล้องกับรายงานก่อนหน้าจากตัวอย่างในภูมิภาคอื่น การศึกษาเซลล์พันธุศาสตร์โดยใช้ลักษณะโพลีทีนโครโมโซมพบว่า *C. striatipennis* มีโพลีทีนโครโมโซม 4 แท่งประกอบด้วยแท่ง AE, CD, BF และ G พบตำแหน่ง nucleolar organizer และ balbiani ring บนแท่ง G โพลีทีนโครโมโซมของ *C. javanus* มี 4 แท่ง แต่ไม่สามารถระบุแขนของโครโมโซมได้ยกเว้นแท่ง Gเนื่องจากคุณภาพของโครโมโซมไม่ดีโพลีทีนโครโมโซมของ *K. tainanus*มี 4 แท่ง พบว่าปลายแท่ง G มีการเชื่อมต่อกับปลายแท่ง E การศึกษาดีเอ็นเอบาร์โค้ดโดยใช้ลำดับนิวคลีโอไทด์ของไมโทคอนเดรียลดีเอ็นเอของยีน cytochrome *c* oxidase subunit I (COI) พบว่า สามารถระบุชนิดได้ถูกต้องทั้งหมด จากการวิเคราะห์สายสัมพันธ์ทางวิวัฒนาการที่พบว่าทั้งหมดแยกเป็นโมโนไฟลิติก (monophyletic) อย่างไรก็ตาม *C.striatipennis* พบว่า แยกเป็นสองกลุ่มชี้ให้เห็นความหลากหลายซ่อนเร้นภายในสปีชีส์ที่ต้องตรวจสอบต่อไป

คำสำคัญ: ริ้นน้ำจืด *Chironomus* ดีเอ็นเอบาร์โค้ด โพลีทีนโครโมโซม

Abstract

The larvae of the family Chironomidae are important components of freshwater ecosystems. However, taxonomic knowledge of these insects is poorly developed in Thailand. In this study we examined multiple character sets for species identification of the larval stage of three Chironomid species, *Chironomus striatipennis*, *C. javanus* and *Kiefferulus tainanus*. Specimens were collected from Maha Sarakham and Roi Et Province, Thailand.The morphological characters of these species agreed with previously published descriptions from other geographic regions. Cytological examinations revealed that *C. striatipennis* has four polytene chromosomes with the arm combinations of AE, CD, BF and G. The nucleolar organizer and Balbiani Ring were located on the chromosome arm G. *C. javanus* has four polytene chromosomes, but arm combinations could not be determined due to the poor quality of the chromosomes except for arm G. *K. tinanus* has four chromosomes,and chromosome arm G was connected to chromosome E. DNA barcoding based on mitochondrial cytochrome *c* oxidase subunit I (COI) sequences perfectly differentiated these species. The results are consistent with phylogenetic analysis that revealed that all three species formed monophyletic clades with strong support. However, two distinct groups were found among the specimens of *C. striatipennis* indicated cryptic diversity in this species that needs further examination.

Keywords: Chironomidae, *Chironomus*, DNA barcode, polytene chromosome

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Introduction

The family Chironomidae contains diverse and abundant macroinvertebrates found in freshwater ecosystems. There are 4,147 species recorded worldwide[1].Larvae of these insects play an important role in freshwater ecosystems[2]because they are important sources of food for fishes and many other aquatic predators[3]. Chironomid larvae have frequently been included in biomonitoring programs because they are sensitive to environmental changesas well as chemical and heavy mental contamina tion[4,5, 6,7]. However, the major obstacle when using Chironomid larvae as bioindicators is a lack of taxonomic background. In Thailand, to the best of our knowledge, there areonly two reports on the species diversity of Chironomidae[8, 9].

Traditional taxonomy of the larva based on morphological characters provides important basic information, but also suffers a major limitation because of the high degree of morphological similarity between closely related species[10].Cytotaxonomy using polytene chromosome banding patterns has also contributed to Chironomidae taxonomy[11]. Nonetheless, using polytene chromosomes for Chironomidae taxonomy requires highly skilled staff and basic information from the morphological taxonomy for species reference.

To overcome the limitations of traditional taxonomy, molecular approaches have been introduced to the taxonomic study of living organisms. DNA barcoding is among the most widely used methods. This technique uses short DNA sequences (500 – 600 bp) to differentiate species based on the level of genetic distance[12]. Several studies on Chironomidae from different geographic regions indicated that the cytochrome *c* oxidase I (COI) sequences were highly effective for species identifications[10, 13, 14, 15, 16, 17].

In this study, we examined morphological, cytological and DNA barcoding sequences of three Chironomids species in Thailand. The aim was to provide taxonomic information of these important insects that could be used in further study.

Materials and Methods

Specimen collection and identification

A total of 18 collections were made from six sampling sites in MahaSarakham and Roi-Et provinces, Northeastern Thailand (Table 1). Larval specimens were preserved in Carnoy's fixative (3:1of 95% ethanol/glacial acetic acid) and stored at -20 °C until processing. Preserving specimens in Carnoy's solution enables us to obtain morphological, cytological and molecular genetic data from the same individual[18]. Environmental conditions of the larval habitats recorded included altitude (m), water conductivity, pH, water temperature, depth and width (Table 1). Species identification and descriptions of the morphological characters followed Epler[19], Cranston[9] and Martin[20].

Cytogenetic study

Fourth instar larvae were used for salivary gland polytene chromosome preparations using the Feulgen stain method[21]. Identification of the chromosome arms followed Keyl[22] and Dévai*et al*[23]. Chromosome arm designations were also made by comparison with a previous publication of *Chironomus circumdatus* Kieffer[24].

DNA extraction, PCR primer amplification and sequencing

Total genomic DNA was extracted using the Genomic DNA extraction mini kit (RBC BioScience, Taiwan). The polymerase chain reaction (PCR) was used to amplify a region of themitochondrial cytochrome *c* oxidase I (COI) gene using the primers LCO1490 (5'-GGTCAAAAATCATAAAGATATTGG-3') andHCO2198 (5'-TA AACTTCAGGGTGACCAAAAAAC-3')[25]. DNA was amplified in a 50 µl reaction containing 10X PCR buffer, 50 mM $MgCl_2$, 10 µM $dNTP_S$, 10 µM of each primer and 5 units *Taq* DNA polymerase (Vivantis, Malaysia). The temperature profile for the PCR reaction included initial denaturation at 94°C for 3 min, followed by 35 cycles of 94ºC for 30 s, 45ºC for 1 min and 72ºC for 1 min, and the final extension at 72ºC for 5 min. PCR products were checked with 1% agarose gel electrophoresis. Successful amplification products were purified using the HiYield™

Gel/PCR Extraction Kit (RBCBIOSCIENCE, Taiwan). Sequencing was performed at Macrogen (Seoul, Korea) using the same primers as in the PCR.

Data analysis

A total of 33 COI sequences were included in the analyses. Of these, 18 were obtained in this study and 15 were from conspecific sequences available in Genbank (Table 1). DNA sequences were aligned using CLUSTAL X[26]. Intraspecific and interspecific genetic divergence values werecalculated based on the Kimura 2-parameter (K2P) model using MEGA 5[27]. The phylogenetic relationships of species were calculated using three methods including maximum parsimony (MP), neigh bor-joining (NJ) and Bayesian analysis. The MP analysis was performed in PAUP*4.0b10[28]. A neighbor-joining (NJ) tree was estimated in MEGA 5[27]. Branch support for MP and NJ trees was calculated based on 1,000 bootstrap replications. The phylogenetic relationship based on the Bayesian method was estimated using MrBayes software[29]. The Bayesian analysis was run for 2,000,000 generations with a sampling frequency at every 100 generations. *Cricotopus tristis* (Genbank accession number DQ865173) was used as the outgroup for all phylogenetic analyses.

Table 1 Details of the sampling sites for Chironomidaes pecimens in the Northeast of Thailand.

Date	Locality	Code	Latitude/longitude	Altitude (m)	Conductivity (μS/cm)	pH	Water temperature (ºC)	Depth (cm)	Width (m)
19/5/2011	Ban nongkham, Kantharawichai District, Maha Sarakham Province	NK1 NK2	16°17' N/ 103°15' E	262	1590	7.05	30.3	1.30	3
27/5/2011	Kantharawichai District, Maha Sarakham Province	KW1 KW2 KW3	16°15' N/ 103°15' E	151	225	8.08	29.9	10	5
23/2/2012	Ban khamriang, Kantharawichai District, Maha Sarakham Province	KR1 KR2 KR3 KR4 KR5	16°15' N/ 103°15' E	160	386	8.64	25.0	19.30	70
10/7/2012	Mahasarakham university Maha Sarakham Province	MSU1 MSU2	15°18' N/ 103°22' E	141	578	8.66	35.6	22	5
19/12/2013	Rajabhat Mahasarakham university Maha Sarakham Province	RMU11 RMU21 RMU22 RMU23 RMU24	16°11' N/ 103°16' E	144	654	7.66	19.22	35	3
2/3/2012	ChaturaphakPhiman District, Roi Et Province	RE	15°53' N/ 103°33' E	163	530	9.88	28.9	40	5

Table 2 List of species and Genbank accession numbers of COI sequences included in this study.

Species	Accession number code	Country of origin
Chironomus striatipennis	AB838642	Japan
C. striatipennis	AB638643	Japan
C. striatipennis	AB838644	Japan
C. striatipennis	AB838645	Japan
C. striatipennis	AB838646	Japan
C. striatipennis	JF412086	Korea
C. striatipennis	JF412087	Korea
C. striatipennis	JF412088	Korea
C. striatipennis	JQ350720	Korea
C. striatipennis	KC407765	Korea
C. striatipennis	KM013389	Thailand
C. striatipennis	KM013390	Thailand
C. striatipennis	KM013391	Thailand
C. striatipennis	KM013392	Thailand
C. striatipennis	KM013393	Thailand
C. striatipennis	KM013394	Thailand
C. striatipennis	KM013395	Thailand
C. javanus	JF412082	Korea
C. javanus	JF412083	Korea
C. javanus	JF412084	Korea
C. javanus	DQ648203	Australia
C. javanus	KM013378	Thailand
C. javanus	KM013379	Thailand
C. javanus	KM013380	Thailand
C. javanus	KM013381	Thailand
C. javanus	KM013382	Thailand
C. javanus	KM013383	Thailand
C. javanus	KM013384	Thailand
C. javanus	KM013385	Thailand
C. javanus	KM013386	Thailand
Kiefferulus tainanus	DQ648225	Australia
K. tainanus	KM013387	Thailand
K. tainanus	KM013388	Thailand

Results

Larval morphology, polytene chromosomes and DNA barcode

Chironomus striatipennis Kieffer

The morphological characters of Thai specimens of *C. striatipennis* (Fig. 1) agree with the description of this species from other geographic regions[20, 30]. This species was found in habitats at elevations of 151-160 m above sea level, water conductivity between 386 - 1,590 µS/cm, pH between 7.05 - 8.64 and water temperature range from 25.0 - 30.3 ºC. Larvae were collected at a depth of about 1.3 - 19.5 cm.

The polytene chromosome of *C. striatipennis* has four pairs (Fig. 2) with the chromosome arm combinations of BF, CD, AE and G. Thus, this species belongs to the pseudothummi-cytocomplex[20]. Chromosomes BF, CD and AE are long and submetacentric while chromosome G is short. The nucleolar organizer (N) and Balbiani ring (BR) were found on chromosome G (Fig. 2).

Seven COI barcoding sequences were obtained from Thai *C. striatipennis*. Ten sequences of *C. striatipennis* from Genbank were included in the analysis. As the phylogenetic analyses revealed two distinct clades among the specimens of *C. striatipennis* the intraspecific and interspecific genetic divergences were also calculated for each clade (Fig. 7). Clade I-1 contained 14 specimens and all of the Thai *C. striatipennis* belonged to this clade. The intraspecific genetic divergence values for the members of this clade ranged between 0% and 3.1% with a mean of 1.1%. The interspecific genetic divergence values ranged from 13.5% to 16.8% with a mean of 15.2%. Three individuals of *C. striatipennis* (two from Japan and one from Korea) formed clade I-2. The intraspecific genetic divergences within this clade ranged from 0.4% to 2.2% with a mean of 1.6%. The interspecific genetic divergences ranged from 10.2% to 16.4% with a mean of 13.1%. Intraspecific genetic divergence values for the combined data of *C. striatipennis* ranged from 0% to 13.8% with a mean of 4.4%. The interspecific genetic divergences ranged from 13.5% to 16.8% with a mean of 15.0% (Table 3).

Chironomus javanus Kieffer

The morphological characters of *C. javanus* (Fig. 3) agreed with a description from Malaysia[30]. This species was found in habitats at an elevation of 141 - 151 m above sea level, water conductivity of 225 - 654 µS/cm, pH between 7.66 - 8.66 and water temperature of 19.22 - 35.6ºC. Larvae were collected at depths of 10 - 35 cm.

C. javanus has four polytene chromosomes. The chromosome arm designations were difficult to determine because of the poor quality of the chromosome bands. Among the seven chromosomes arms, only chromosome arm G was certainty identifiable (Fig. 4). A prominent nucleolus organizer and two Balbiani rings were found on chromosome arm G (Fig. 4).

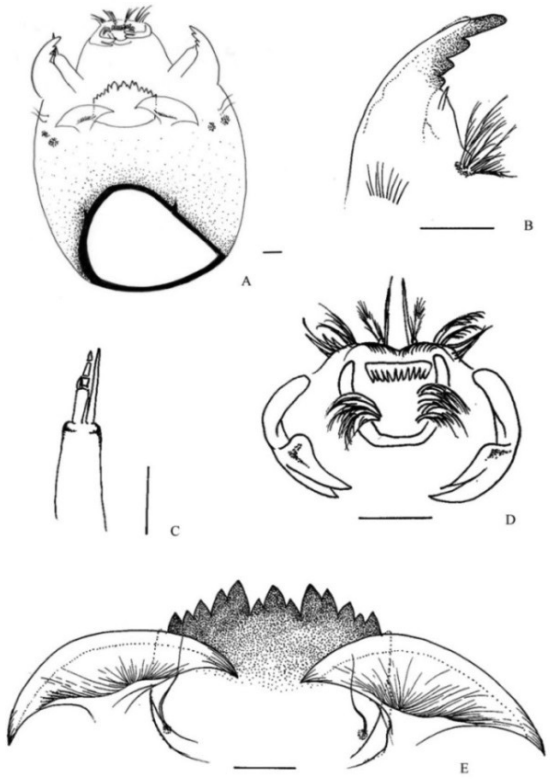

Figure 1 Larval morphology of *Chironomus striatipennis*; A - head capsule, B - mandible, C - antenna, D - labrum and E - ventomental plate. Scale bar represents 50 µm.

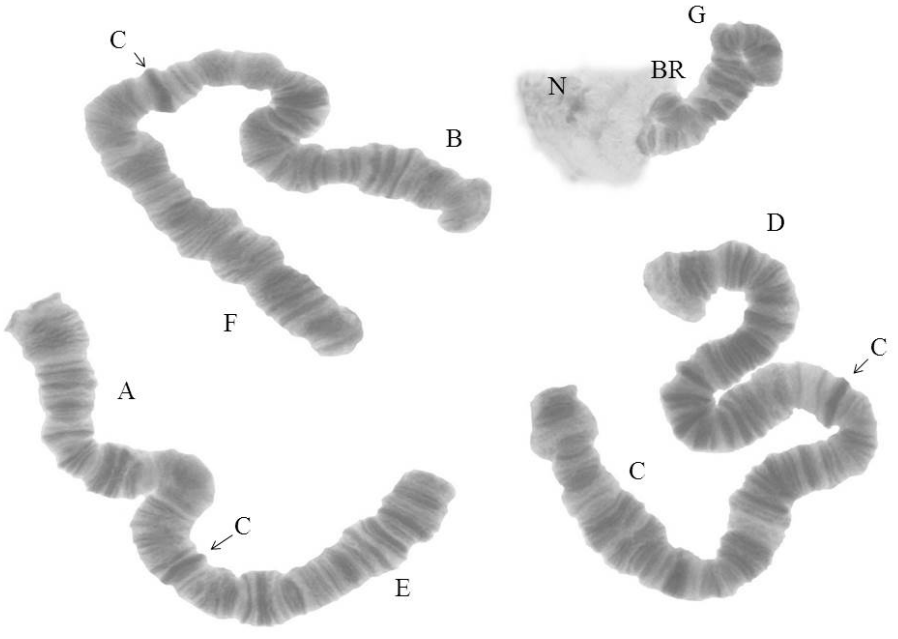

Figure 2 Polytene chromosome of *Chironomus striatipennis*.C, centromere; BR, Balbiani Ring.

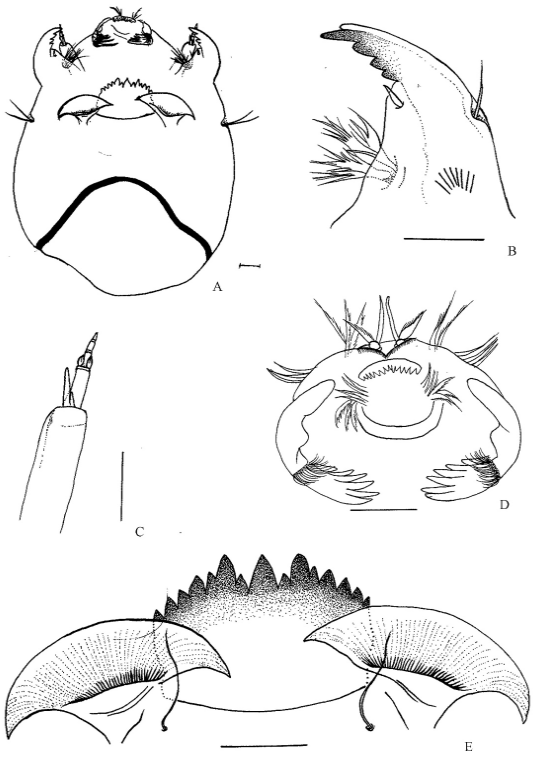

Figure 3 Larval morphology of *Chironomus javanus*; A - head capsule, B - mandible, C - antenna, D - labrum and E - ventomental plate. Scale bar represents 50 µm.

Nine COI barcoding sequences were obtained from *C. javanus* in Thailand and four (three from Korea and one from Australia) from Genbank were included. The intraspecific genetic divergence of the combined specimens ranged from 0% to 1.30% with an average value of 0.70%. The interspecific genetic divergences ranged from 13.3% to 16.8% with a mean of 15.2% (Table 3).

Kiefferulus tainanus Kieffer

The morphological characters of the larval stage of Thai *K. tainanus* are shown in Fig. 5. This species was collected from habitats at an elevation of 144 - 163 m above sea level, water conductivity of 530 - 654 µS/cm, pH between 7.66 - 9.88 and water temperature at 19.22 - 28.9 ºC. Larvae were collected at a water depth of approximately 35 - 40 cm.

K. tainanus has four polytene chromosomes with the arm combinations of AE, CD, FB and G. However, chromosome arm G in most specimens examined was connected to chromosome arm E. A prominent nucleolar organizer was found at the terminal of chromosome arm G (Fig. 6).

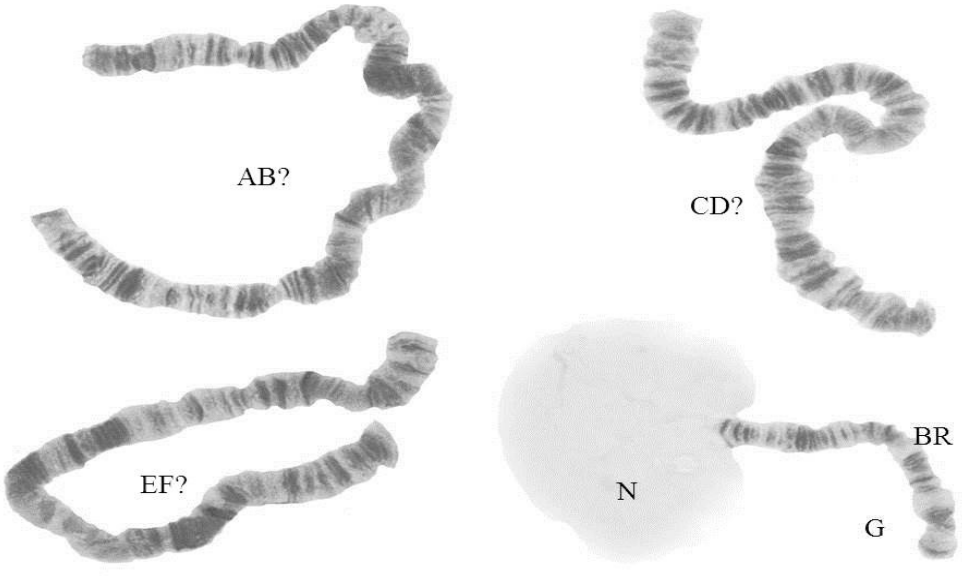

Figure 4. Polytene chromosome of *Chironomus javanus.*C, centromere; N, nucleolar organizer; BR, Balbiani Ring.

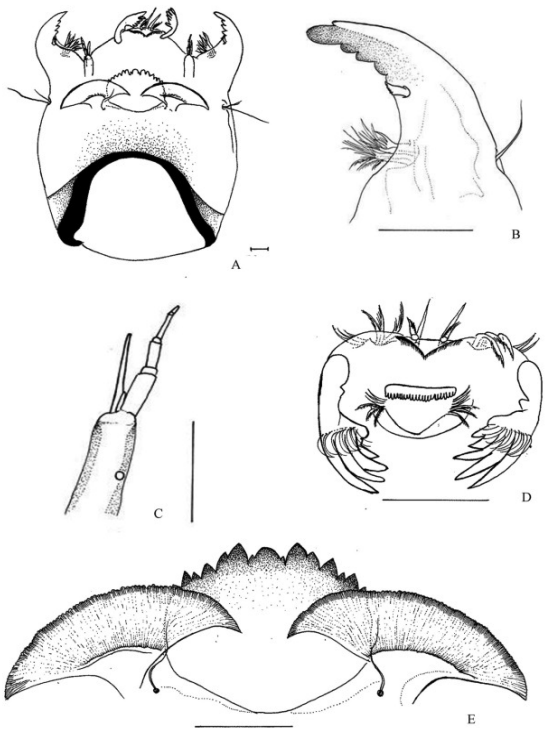

Figure 5 Larval morphology of *Kiefferulus tainanus*; A - head capsule, B - mandible, C - antenna, D - labrum and E - ventomental plate. Scale bar represents 50 μm.

The COI sequences of this species were obtained from two Thai specimens. A single COI sequence of this species from Genbank was included in the analysis. The intraspecific genetic divergences ranged between 0.7% and 3.1% with a mean of 2.1%. The interspecific genetic divergences ranged from 15.4% to 16.8% with a mean of 16.1% (Table 3).

Phylogenetic relationships

All three phylogenetic methods (NJ, MP and Bayesian) revealed nearly identical tree topologies. Thus, only the NJ tree is shown (Fig. 7). There were two main clades among the specimens included in the analyses. Clade I comprised specimens of *C. striatipennis* and *C. jarvanus*. Both species formed a monophyletic clade with strong support. The sequences of *C. striatipennis* divided into two subclades (I-1 and I-2). Most specimens belonged to subclade I-1. Three specimens (two from Japan and one from Korea) comprised clade I-2. The genetic diver gence between these two subclades was high (11.6%), suggesting that they might represent different species. Three specimens of *K. tainanus* (two from Thailand and one from Australia) formed clade II with strong support.

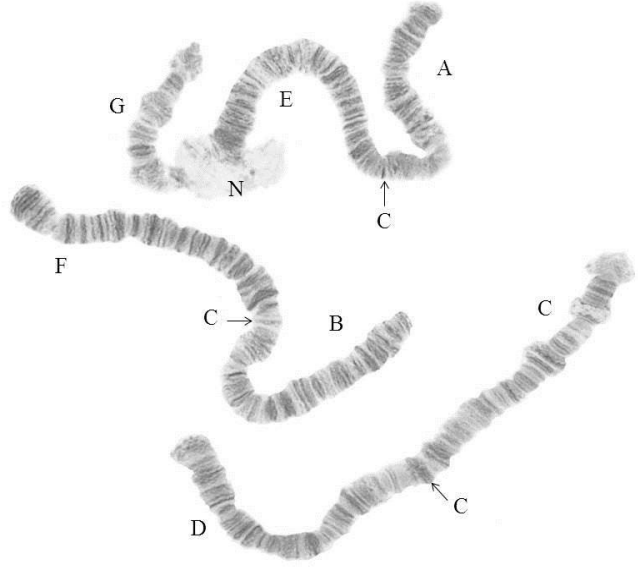

Figure 6 Polytene chromosome of *Kiefferulus tainanus*.C,centromere; N, nucleolar organizer; BR, Balbiani Ring.

Discussion

Chironomid larvae are important components of freshwater ecosystems and are valuable for biomonitoring programs[5]. Thus, taxonomic knowledge is crucial for understanding all aspects of these insects. Traditional taxonomy ofthe larval stage suffers the major obstacle of the high degreeof morphological homogeneity[10]. In Thailand, there have been few taxonomic reports of the Chironomidae[8, 9]. Therefore, the morphological characters of the larvae provided in the present study will significantly contribute to current knowledge of Chironomidae diversity in Thailand.

Polytene chromosomes are important taxo nomic tools for Chironomidae[11, 31, 32]. Different species usually possess different banding patterns, which enables precise species identification[31]. However, the difficulty of using polytene chromosomes as a taxonomic tool for Chironomidae is the lack of other previously identified morphological characters of the species. Integrating morphological characters with polytene chromosomes would thus enable multiple character sets for species identification. This is particularly useful for the differentiation of closely related species that often shown great morpho logical similarity[10].

In addition to the morphology and cytology, we also provide the DNA barcoding sequences ofthese species. DNA barcode has been usedsuccessfully to delimit species of the Chironomidae[33, 34]. Our results revealed that DNA barcoding sequences performed well when differentiating the three species included in this analyses. All three species were perfectly differentiated from each other and formed well-supported monophyletic clades in the phylogenetic analyses.

As well as species identification, DNA barcoding is also useful for uncovering cryptic diversity[13]. Our results revealed two distinct clades among the specimens of *C. striatipennis*. All Thai specimens of *C. striatipennis* formed clade I-1 with some specimens from Japan and Korea, while three specimens (two from Japan and one from Korea) formed another clade (I-2). The level of genetic divergence among these clade was high (11.6%) that indicated they most likely belong to different species. Further study is needed to clarify this situation.

Table 3 Mean and range of intraspecific and interspecific genetic divergence of the COI sequences for three Chironomidae species based on the Kimura 2-parameter model.

Species	N	Range of intraspecific genetic diver-gence (mean)	Range of interspecific genetic diver-gence (mean)
C. striatipennis clade I-1	14	0-0.031 (0.011)	0.135-0.168 (0.152)
clade I-2	3	0.004-0.022 (0.016)	0.102-0.164 (0.131)
All	17	0-0.138 (0.044)	0.135-0.168 (0.150)
C. javanus	13	0-0.013 (0.007)	0.133-0.168 (0.152)
K. tainanus	3	0.007-0.031(0.021)	0.154-0.168 (0.161)

N represents number of COI sequence.

Conclusion

In this study we provided multiple character sets (morphology, cytology and DNA barcodes) for species identification of three Chironomiidae in Thailand. Integrating these taxonomic tools enables straightforward species recognition and will enhance further study of these insects. We also identified cryptic diversity in *C. striatipennis* based on DNA barcoding sequences that has not previously been detected using traditional taxonomy. Therefore, the results highlight the significance of integrating multilevel taxonomic tools for fully understanding Chironomiidae biodiversity.

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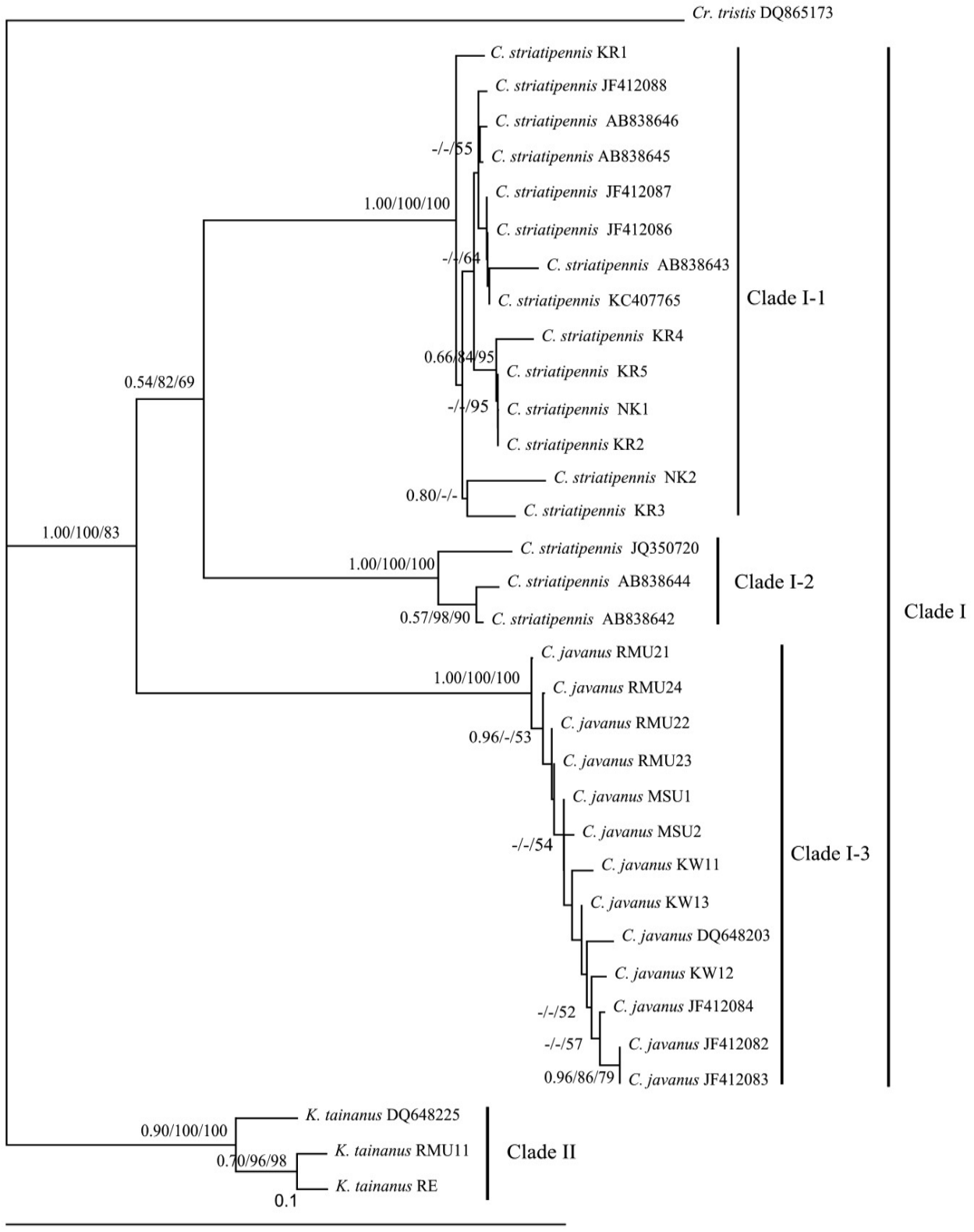

Figure 7 Neighbor joining trees for cytochrome *c* oxidase I (COI) sequences of the three species of Chironomidae. Posterior probability for Bayesian analysis and bootstrap values for neighbor joining and maximum parsimony are shown above the branch. Scale bar represents 0.1 substitutions per nucleotide position.

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