

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

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บทคัดย่อ

ริ้น้ำจืดเป็นแมลงในวงศ์ Chironomidae มีความสำคัญต่อระบบนิเวศน้ำจืด อย่างไรก็ตามข้อมูลอนุกรมวิธานของริ้น้ำจืดในประเทศไทยยังมีการศึกษาไม่มาก ในการศึกษาที่ใช้ลักษณะในการศึกษาอนุกรมวิธานหลายระดับเพื่อใช้ในการจำแนกชนิดของริ้น้ำจืด 3 สปีชีส์ได้แก่ *Chironomus striatipennis*, *C. javanus* และ *Kiefferulus tainanus* โดยเก็บตัวอย่างริ้น้ำจืดจากแหล่งน้ำในจังหวัดมหาสารคามและจังหวัดร้อยเอ็ด ผลการศึกษาพบว่าลักษณะสัณฐานวิทยาของริ้น้ำจืดทั้ง 3 สปีชีส์สอดคล้องกับรายงานก่อนหน้าจากตัวอย่างในภูมิภาคอื่น การศึกษาเซลล์พันธุศาสตร์โดยใช้ลักษณะโพลีทีนโครโมโซมพบว่า *C. striatipennis* มีโพลีทีนโครโมโซม 4 แห่งประกอบด้วยแขนง AE, CD, BF และ G พบตำแหน่ง nucleolar organizer และ balbiani ring บนแขนง G โพลีทีนโครโมโซมของ *C. javanus* มี 4 แขนง แต่ไม่สามารถระบุแขนงของโครโมโซมได้ยกเว้นแขนง G เนื่องจากคุณภาพของโครโมโซมไม่ดี โพลีทีนโครโมโซมของ *K. tainanus* มี 4 แขนง พบว่าปลายแขนง G มีการเชื่อมต่อกับปลายแขนง E การศึกษาดีเอ็นเอบาร์โค้ดโดยใช้ลำดับนิวคลีโอไทด์ของไมโทคอนเดรียลดีเอ็นเอของยีน cytochrome c oxidase subunit I (COI) พบว่าสามารถระบุชนิดได้ถูกต้องทั้งหมด จากการวิเคราะห์สายสัมพันธ์ทางวิวัฒนาการที่พบว่าทั้งหมดแยกเป็นโมโนไฟลิติก (monophyletic) อย่างไรก็ตาม *C. striatipennis* พบว่า แยกเป็นสองกลุ่มชี้ให้เห็นความหลากหลายซ่อนเร้นภายในสปีชีส์ที่ต้องตรวจสอบต่อไป

คำสำคัญ: ริ้น้ำจืด *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. tainanus* 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 worldwide¹. Larvae of these insects play an important role in freshwater ecosystems² because they are important sources of food for fishes and many other aquatic predators³. Chironomid larvae have frequently been included in biomonitoring programs because they are sensitive to environmental changes as well as chemical and heavy metal contamination^{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 are only 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 species¹⁰. Cytotaxonomy using polytene chromosome banding patterns has also contributed to Chironomidae taxonomy¹¹. 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 distance¹². 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 (3:1 of 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 individual¹⁸. 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 Epler¹⁹, Cranston⁹ and Martin²⁰.

Cytogenetic study

Fourth instar larvae were used for salivary gland polytene chromosome preparations using the Feulgen stain method²¹. Identification of the chromosome arms followed Keyl²² and Dévalet *al*²³. Chromosome arm designations were also made by comparison with a previous publication of *Chironomus circumdatus* Kieffer²⁴.

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 the mitochondrial cytochrome *c* oxidase I (COI) gene using the primers LCO1490 (5'-GGTCAAAAATCATAAAGATATTGG-3') and HCO2198 (5'-TA AACTTCAGGGTGACCAAAAAAC-3')²⁵. DNA was amplified in a 50 µl reaction containing 10X PCR buffer, 50 mM MgCl₂, 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 94°C for 30 s, 45°C for 1 min and 72°C for 1 min, and the final extension at 72°C 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 X²⁶. Intraspecific and interspecific genetic divergence values were calculated based on the Kimura 2-parameter (K2P) model using MEGA 5²⁷. The phylogenetic relationships of species were calculated using three

methods including maximum parsimony (MP), neighbor-joining (NJ) and Bayesian analysis. The MP analysis was performed in PAUP*4.0b10²⁸. A neighbor-joining (NJ) tree was estimated in MEGA 5²⁷. 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 software²⁹. 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 Chironomidae specimens in the Northeast of Thailand.

Date	Locality	Code	Latitude/longitude	Altitude (m)	Conductivity ($\mu\text{S/cm}$)	pH	Water temperature ($^{\circ}\text{C}$)	Depth (cm)	Width (m)
19/5/2011	Ban nongkham, Kantharawichai District, Maha Sarakham Province	NK1	16°17' N/ 103°15' E	262	1590	7.05	30.3	1.30	3
		NK2							
27/5/2011	Kantharawichai District, Maha Sarakham Province	KW1	16°15' N/ 103°15' E	151	225	8.08	29.9	10	5
		KW2							
		KW3							
23/2/2012	Ban khamriang, Kantharawichai District, Maha Sarakham Province	KR1	16°15' N/ 103°15' E	160	386	8.64	25.0	19.30	70
		KR2							
		KR3							
		KR4							
		KR5							
10/7/2012	Mahasarakham university, Maha Sarakham Province	MSU1	15°18' N/ 103°22' E	141	578	8.66	35.6	22	5
		MSU2							
19/12/2013	Rajabhat Mahasarakham university, Maha Sarakham Province	RMU11	16°11' N/ 103°16' E	144	654	7.66	19.22	35	3
		RMU21							
		RMU22							
		RMU23							
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
<i>Chironomus striatipennis</i>	AB838642	Japan
<i>C. striatipennis</i>	AB638643	Japan
<i>C. striatipennis</i>	AB838644	Japan
<i>C. striatipennis</i>	AB838645	Japan
<i>C. striatipennis</i>	AB838646	Japan
<i>C. striatipennis</i>	JF412086	Korea
<i>C. striatipennis</i>	JF412087	Korea
<i>C. striatipennis</i>	JF412088	Korea
<i>C. striatipennis</i>	JQ350720	Korea
<i>C. striatipennis</i>	KC407765	Korea
<i>C. striatipennis</i>	KM013389	Thailand
<i>C. striatipennis</i>	KM013390	Thailand
<i>C. striatipennis</i>	KM013391	Thailand
<i>C. striatipennis</i>	KM013392	Thailand
<i>C. striatipennis</i>	KM013393	Thailand
<i>C. striatipennis</i>	KM013394	Thailand
<i>C. striatipennis</i>	KM013395	Thailand
<i>C. javanus</i>	JF412082	Korea
<i>C. javanus</i>	JF412083	Korea
<i>C. javanus</i>	JF412084	Korea
<i>C. javanus</i>	DQ648203	Australia
<i>C. javanus</i>	KM013378	Thailand
<i>C. javanus</i>	KM013379	Thailand
<i>C. javanus</i>	KM013380	Thailand
<i>C. javanus</i>	KM013381	Thailand
<i>C. javanus</i>	KM013382	Thailand
<i>C. javanus</i>	KM013383	Thailand
<i>C. javanus</i>	KM013384	Thailand
<i>C. javanus</i>	KM013385	Thailand
<i>C. javanus</i>	KM013386	Thailand
<i>Kiefferulus tainanus</i>	DQ648225	Australia
<i>K. tainanus</i>	KM013387	Thailand
<i>K. tainanus</i>	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 $\mu\text{S/cm}$, pH between 7.05 - 8.64 and water temperature range from 25.0 - 30.3 °C. 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-cytocomplex²⁰. 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 Malaysia³⁰. This species was found in habitats at an elevation of 141 - 151 m above sea level, water conductivity of 225 - 654 $\mu\text{S}/\text{cm}$, pH between 7.66 - 8.66 and water temperature of 19.22 - 35.6°C. 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 certainly identifiable (Fig. 4). A prominent nucleolus organizer and two Balbiani rings were found on chromosome arm G (Fig. 4).

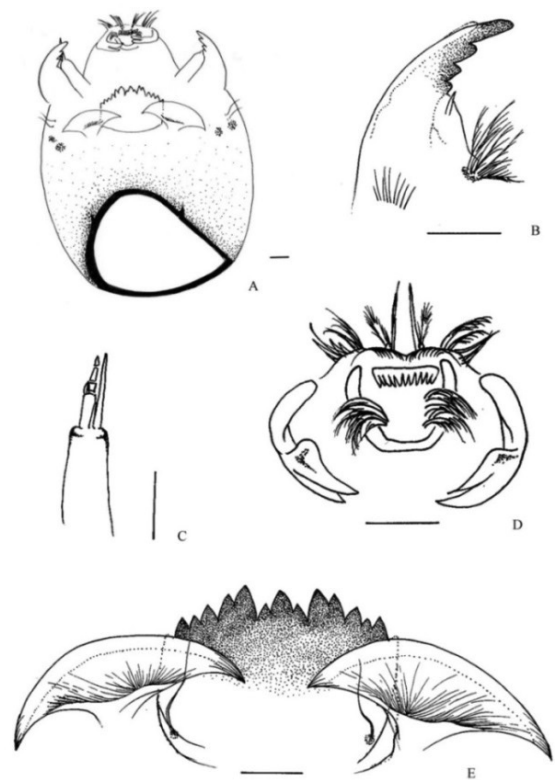


Figure 1 Larval morphology of *Chironomus striatipennis*; A - head capsule, B - mandible, C - antenna, D - labrum and E - ventomental plate. Scale bar represents 50 μm .

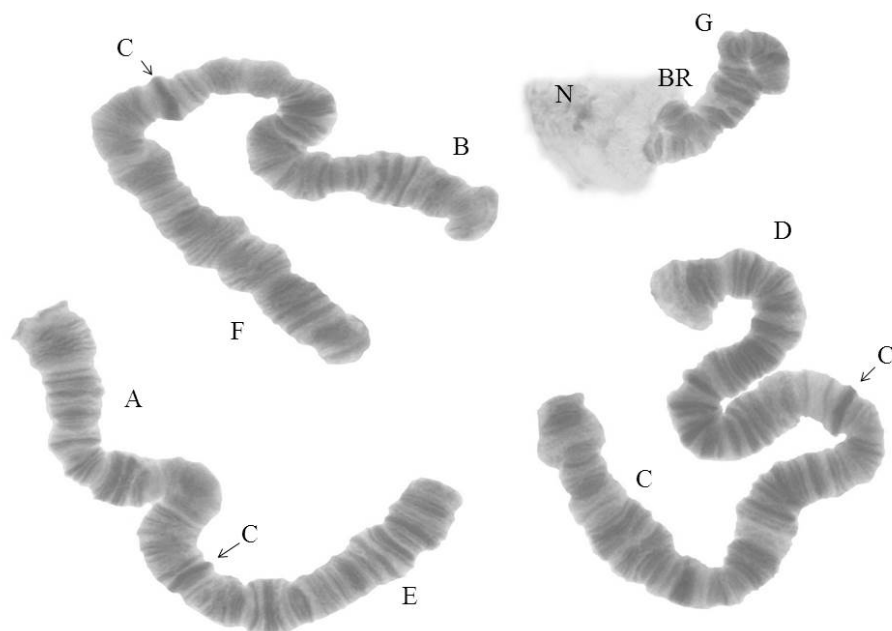


Figure 2 Polytene chromosome of *Chironomus striatipennis*. C, centromere; BR, Balbiani Ring.

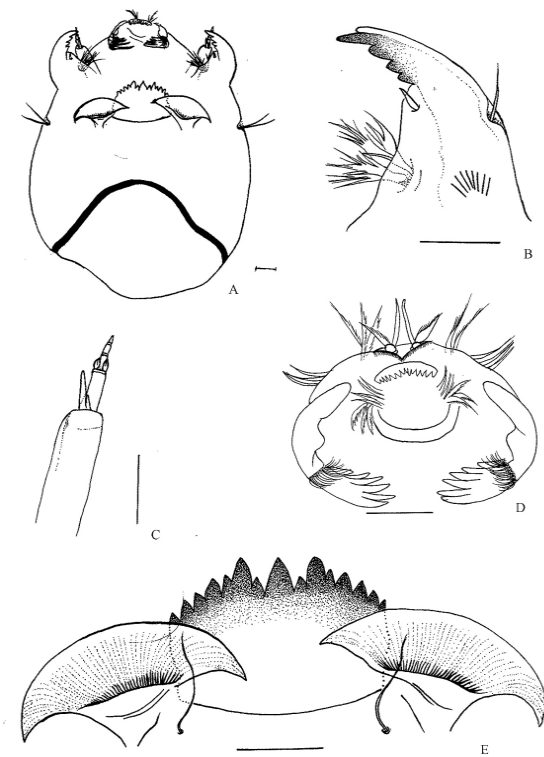


Figure 3 Larval morphology of *Chironomus javanus*; A - head capsule, B - mandible, C - antenna, D - labrum and E - ventomental plate. Scale bar represents 50 μm .

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 $\mu\text{S/cm}$, pH between 7.66 - 9.88 and water temperature at 19.22 - 28.9 $^{\circ}\text{C}$. 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).

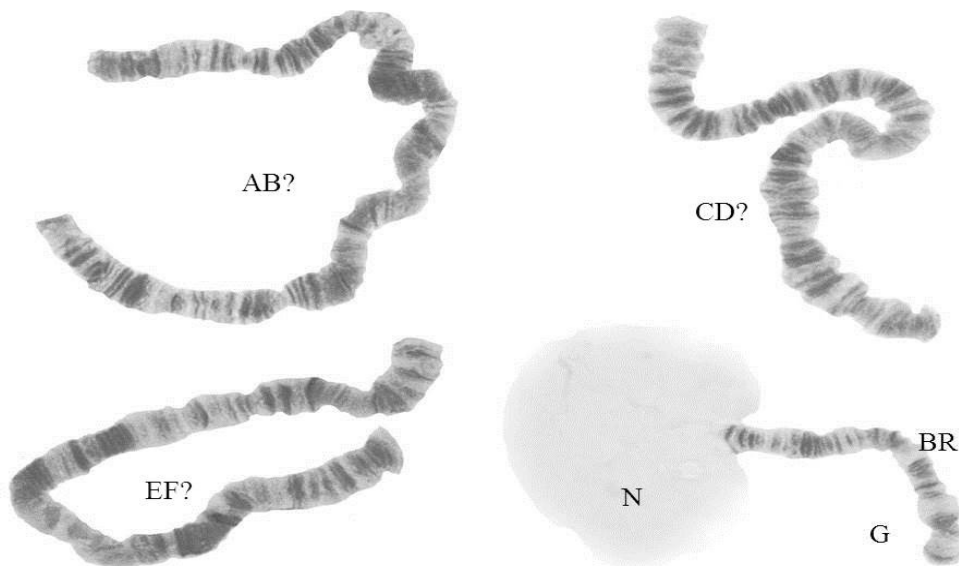


Figure 4. Polytene chromosome of *Chironomus javanus*. C, centromere; N, nucleolar organizer; BR, Balbiani Ring.

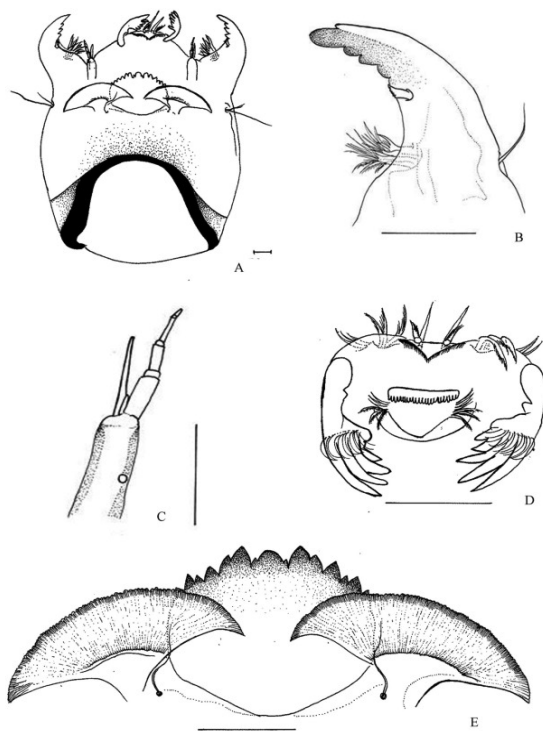


Figure 5 Larval morphology of *Kiefferulus tainanus*; A - head capsule, B - mandible, C - antenna, D - labrum and E - ventromental plate. Scale bar represents 50 µm.

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 divergence 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.

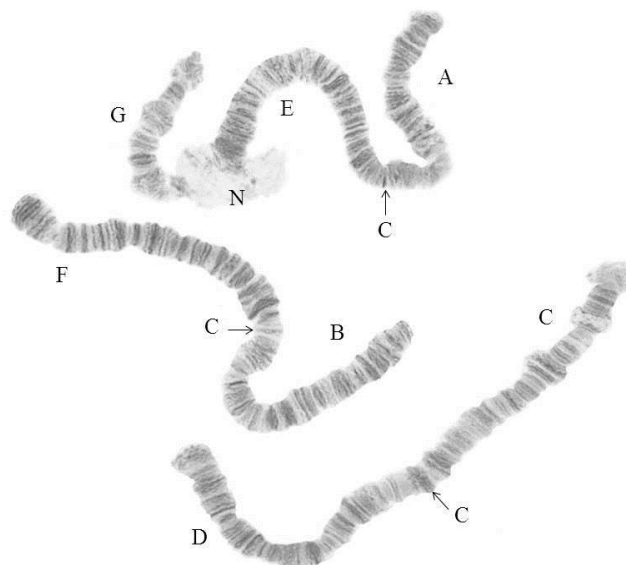


Figure 6 Polytene chromosome of *Kiefferulus tainanus*. C, centromere; N, nucleolar organizer; BR, Balbiani Ring.

Discussion

Chironomid larvae are important components of freshwater ecosystems and are valuable for biomonitoring programs⁵. Thus, taxonomic knowledge is crucial for understanding all aspects of these insects. Traditional taxonomy of the larval stage suffers the major obstacle of the high degree of morphological homogeneity¹⁰. 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 taxonomic tools for Chironomidae^{11, 31, 32}. Different species usually possess different banding patterns, which enables precise species identification³¹. 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 morphological similarity¹⁰.

In addition to the morphology and cytology, we also provide the DNA barcoding sequences of these species. DNA barcode has been used successfully 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 diversity¹³. 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 divergence (mean)	Range of interspecific genetic divergence (mean)
<i>C. striatipennis</i>	14	0-0.031 (0.011)	0.135-0.168
clade I-1			(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)
<i>C. javanus</i>	13	0-0.013 (0.007)	0.133-0.168 (0.152)
<i>K. tainanus</i>	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 Chironomidae 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 Chironomidae biodiversity.

Acknowledgements

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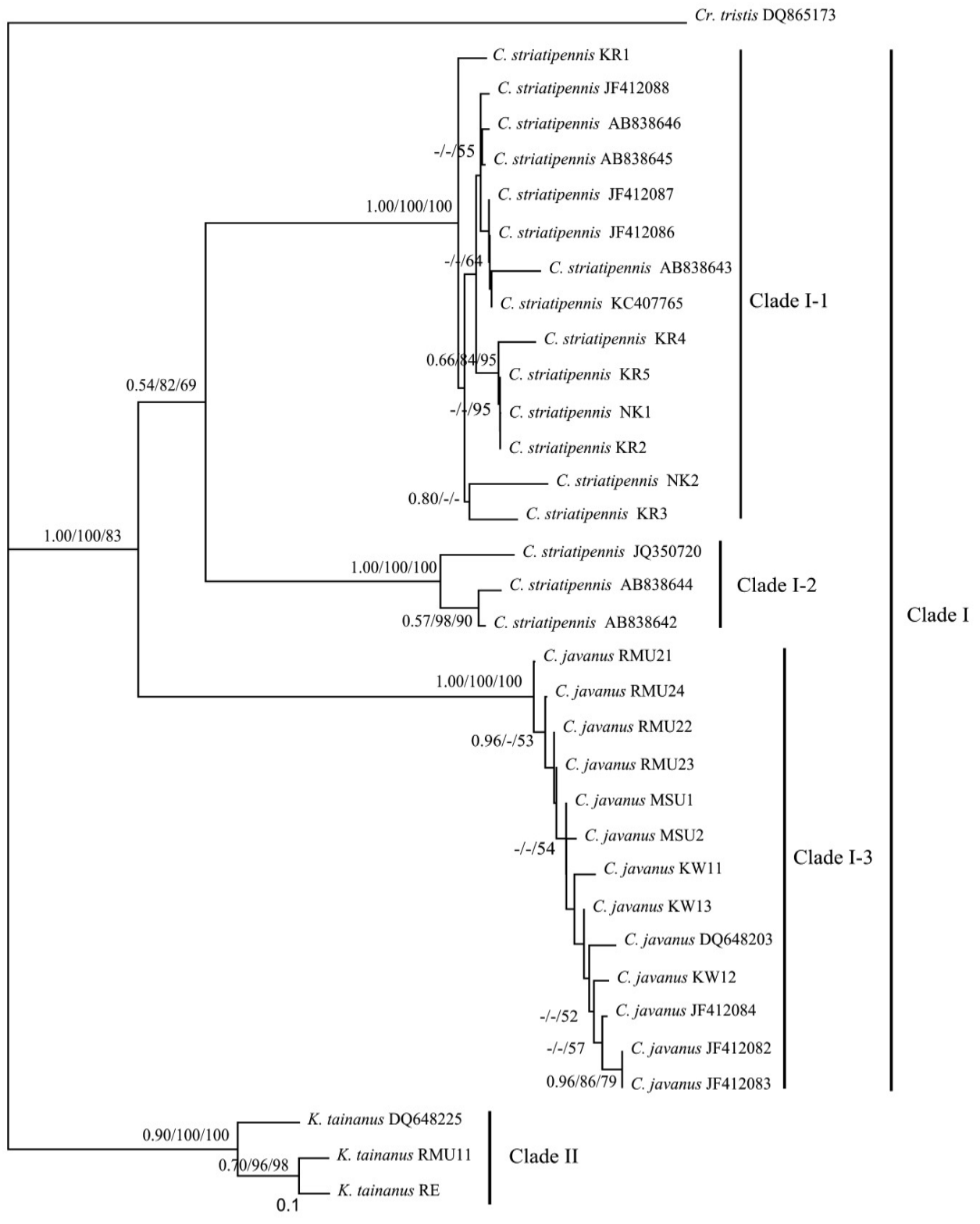


Figure 7 Neighbor joining trees for cytochrome c oxidase I (COI) sequences of the three species of Chironomidae. Posterior probability for Bayesian analysis and bootstrap values for neighbor joining and maximum parsimony are shown above the branch. Scale bar represents 0.1 substitutions per nucleotide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

Reference

- Leonard C, Ferrington J. (2008). Global diversity of non-biting midges (Chironomidae; Insecta-Diptera) in freshwater. *Hydrobiologia*, 595:447–455.
- Ekrem T, Willassen E. (2004). Exploring Tanytarsini relationships (Diptera: Chironomidae) using mitochondrial COII gene sequences. *Insect Systematics and Evolution*, 35:263-276.
- Armitage PD, Cranston PS, Pinder LCV. (1995). *The Chironomidae: The biology and ecology of non-biting midges*. Chapman and Hall, London. 339-364 pp.
- Clements WH, Cherry DS, Cairns J. (1988). Impact of heavy metals on insect communities in streams: A comparison of observational and experimental results. *Canadian journal of fisheries and aquatic sciences*, 45:2017-2025.
- Rosenber DM. (1992). Freshwater biomonitoring and Chironomidae. *Netherland Journal of Aquatic Ecology*, 26(2-4): 101-122.
- Madden CP, Suter PJ, Nicholson BC, Austin AD. (1992). Deformities in Chironomid larvae as indicators of pollution (Pesticide) stress. *Netherlands Journal of Aquatic Ecology*, 26: 551-557.
- Michailova P, Pretova N, Ilkova J, Bovero S, Brunetti S, White K, Sella G. (2006). Genotoxic effect of copper on salivary gland polytene chromosomes of *Chironomus riparius* Meigen 1804 (Diptera, Chironomidae). *Environmental Pollution*, 144:647–654.
- Hashimoto H, Wongsiri T, Wongsiri N, Tirawat C, Lewvanich A, Yasamatsu H. (1981). Chironomidae from rice fields of Thailand with descriptions of 7 new species. *Technical Bulletin, Entomology and Zoology Division*, Department of Agriculture, Bangkok, Thailand, 7: 1-47.
- Cranston PS. (2007). The Chironomidae larvae associated with the tsunami-impacted water bodies of the coastal plain of south-western Thailand. *Bulletin of the Raffles Museum*, 55: 231–244.
- Sharley DJ, Pettigrew V, Parsons YV. (2004). Molecular identification of *Chironomus* spp. (Diptera) for biomonitoring of aquatic ecosystems. *Australian Journal of Entomology*, 43:359-365.
- Martin J. (1979). Chromosomes as tools in taxonomy and phylogeny of Chironomidae (Diptera). *Entomologica Scandinavica*, 10: 67-74.
- Hebert PDN, Cywinska A, Ball SL, Waard JR. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society: B*, 270: 313–322.
- Pfenninger M, Nowak C, Kley C, Steinke D, Streit B. (2007). Utility of DNA taxonomy and barcoding for the inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species. *Molecular Ecology*, 16: 1957-1968.
- Ekrem T, Elisabeth S, Hebert PDN. (2010). Female do count: documenting Chironomidae (Diptera) species diversity using DNA barcoding. *Organisms Diversity and Evolution*, 10: 397-408.
- Carew ME, Pettigrove V, Hoffmann AA. (2005). The utility of DNA markers in classical taxonomy: using cytochrome oxidase I markers to differentiate Australian *Cladopelma* (Diptera: Chironomidae) midges. *Annals of the Entomological Society of America*, 98: 587–594.
- Carew ME, Pettigrove V, Cox RL, Hoffmann AA. (2007). DNA identification of urban Tanytarsinichironomids (Diptera: Chironomidae). *Journal of the North American Benthological Society*, 26: 587–600.
- Al-Shami SA, Siti MN, MdRawi CS, Ahmad AH. (2009). Preliminary study of phylogenetic relationship of rice field Chironomidae (Diptera) inferred from DNA sequences of mitochondrial cytochrome oxidase subunit I. *American Journal of Applied Sciences*, 6: 1004-1009.
- Pramual P, Kuvangkadilok C, Baimai V, Walton C. (2005). Phylogeography of the black fly *Simulium tani* (Diptera: Simuliidae) from Thailand as inferred from mitochondrial DNA sequences. *Molecular Ecology*, 13: 3989-4001.
- Epler JH. (2001). *Identification manual for the larval Chironomidae (Diptera) of North and South Carolina*. North Carolina, USA.
- Martin J. (2011). *Morphology and cytology of Oriental (Indomalayan Realm) Chironomus species*. Department of Genetics, The University of Melbourne, Australia.

21. Rothfels KH, Dunbar RW. (1953). The salivary gland chromosomes of the black fly, *Simulium vittatum* Zett. *Canadian Journal of Zoology*, 31:226-241.
22. Keyl HG. (1962). Chromosomen evolution bei *Chironomus* II. Chromosomenumbauten and phylogenetische Beziehungen der Arten. *Chromosoma*, 13: 464-514.
23. Devai Gy, Miskolczi M, Wülker W. (1989). Standardization of chromosome arms B, C and D in *Chironomus* (Diptera, Chironomidae). Advances in Chironomidology. Part I. *Acta Biologica Debrecina Supplementum Oecologica Hungarica*, 2:79-92.
24. Pramual P, Gomontean B, Buasay V, Sriksamwiang N, Suebkar P, Niamlek C, Donsinphoem Y, Chalatchiao K. (2009). Population cytogenetics of *Chironomus circumdatus* Kieffer, 1921 (Diptera, Chironomidae) from Thailand. *Genetica*, 135: 51-57.
25. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3: 294-299.
26. Thompson JD, Gibson TJ, Plewniak F, Jeanmouging F, Higgins DG. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 24:4876-4882.
27. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28: 2731-2739.
28. Swofford DL. (2002). PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.
29. Huelsenbeck JP, Ronquist F. (2001). MRBAYES: Bayesian inference of phylogenetic trees, Article, *Bioinformatics*, 17(8):754-755.
30. Al-Shami SA, Rawi CSM, Ahmad AH, Nor SAM. (2012). Redescription of *Chironomus javanus* and *Chironomus kiiensis* (Diptera: Chironomidae) Larvae and Adults Collected from a Rice Field in Pulau Pinang, Malaysia. *Tropical Life Sciences Research*, 23(1): 77-86.
31. Martin J, Andreeva EN, Kiknadze II, Wülker WF. (2006). Polytene chromosomes and phylogenetic relationships of *Chironomus atrelela* (Diptera: Chironomidae) in North America. *Genome*, 49: 1384 – 1392.
32. Wülker WF, Kiknadze II, Istomina AG. (2011). Karyotypes of *Chironomus* Meigen (Diptera: Chironomidae) species from Africa. *Comparative Cytogenetics*, 5(1): 23 – 46.
33. Kim S, Song KH, Ree HI, Kim W. (2012). A DNA barcode library for Korean Chironomidae (Insecta: Diptera) and indexes for defining barcode gap. *Molecules and Cells*, 33(1), 9-17.
34. Brodin Y, Ejdung G, Strandberg J, Lyrholm T. (2013). Improving environmental and biodiversity monitoring in the Baltic Sea using DNA barcoding of Chironomidae (Diptera). *Molecular Ecology Resources*, 13(6): 996-1004.