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Efficiency of DNA Barcodes for Identification and Documenting Aquatic Insect Diversity in Rice Fields

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ABSTRACT.– Rapid and accurate identifications are crucial for biodiversity assessment. Yet, traditional methods for species identification have some limitations. In this study, we tested the efficiency of mitochondrial cytochrome *c* oxidase I barcoding sequences for species identification and documenting diversity of aquatic insects in the rice fields of Thailand. Considerable success rate (80%) for species identification was found among the species of the order Odonata. Unidentifiable specimens of immatures were successfully associated with conspecific adults or by matching with reference sequences in the public DNA barcoding library. However, some specimens were ambiguous, possibly due to incomplete lineage sorting of closely related species or erroneous identification of the sequences in the public database. The technique was less successful for other insect orders because a lack of reference sequences in the DNA barcode library limits the utility of DNA barcoding. The Poisson tree process and Automatic Barcode Gap Discovery species delimitations revealed that the number of species recognized is more than twice that based on morphological identification. Therefore, DNA barcoding has potential for use in species identification and biodiversity assessment of the aquatic insects in the rice field ecosystem.

KEY WORDS: biodiversity, COI, rice field, species delimitation

INTRODUCTION

Rice fields are an important artificial wetland ecosystem possessing different categories of the environmental conditions and providing habitats for different faunas (Lawler, 2001; Bambaradeniya and Amerasinghe, 2003). Diverse groups of organisms from microbial, fungi, plankton and macroinvertebrates to large vertebrates such as fishes, snakes and birds utilize this artificial ecosystem (Edirisinghe and Bambaradeniya, 2010). It has been found that biological diversity is positively related with the productivity of agricultural ecosystems (Roger et al., 1991; Scherr and McNeely, 2008; Tscharntke et al., 2012). However, using chemical pesticides and other inappropriate managements could reduce biodiversity in agricultural ecosystems (Wilson et al., 2008; Watanabe et al., 2013). Thus, information regarding species diversity in the rice field is needed for monitoring as well as appropriate management.

Aquatic insects are among the major components of the rice field faunas and play diverse roles in this ecosystem. For example, members of the order Odonata are the major predator while the orders Diptera, Coleoptera and Lepidoptera are largely pests of the rice (Bambaradeniya et al., 2004). In addition, aquatic insects could also have applications as bioindicators of the ecosystem (Federico Rizo-Patrón et al., 2013). Therefore, understanding taxonomy and biodiversity of these insects is important for applications as well as appropriate and

sustainable management of the rice field ecosystem.

The traditional method of species identification based on morphological characters has some limitations such as incomplete taxonomic knowledge, high intraspecific morphological variation, a lack of valid morphological characters for species recognition of particular life stages and damaged specimens. Beside these limitations, insufficient expertise or lack of an expert who has experience in the taxonomy of relevant insect groups also present major obstacles. One possible way to solve these issues is using the DNA barcode. Although there are also some limitations of using molecular genetic data for species identification (Frézal and Leblois, 2008) this method has more advantages compared to traditional taxonomy (Sweeney et al., 2011, Gill et al., 2013). DNA barcoding has been used successfully for species identification and documenting biodiversity of many insect groups (Rach et al., 2008; Damm et al., 2010; Jinbo et al., 2011; Sweeney et al., 2011; Jackson et al., 2014). Thus, this DNA marker can potentially be used for identification and documenting species diversity of rice field aquatic insects.

Thailand is the major rice exporting country. Approximately 18% (about 9 million hectares) of the land is used for rice cultivation (http://www.ricethailand.go.th). However, data on biodiversity of the insects in rice fields is scanty due largely to the lack of the taxonomic knowledge. In this study, we used DNA barcoding sequences to test the efficiency of this molecular marker for species identification using public DNA barcode libraries (i.e. BOLD and GenBank). We also used the species delimitation approach based on the mitochondrial cytochrome c oxidase I (COI) sequence to document species diversity in the rice field ecosystem in northeastern Thailand.

MATERIALS AND METHODS

Specimen collection and identification

Insects were collected from the eight rice fields in four provinces in northeastern Thailand between September and November 2017 (Table 1). Specimens were collected using the kick-sampling method with a 500-μm mesh hand net. Adult flying insects were collected using a sweep net along a line transect across the rice field. To compare the efficiency of DNA barcoding with morphological taxonomy in real practice (i.e. when monitoring / examining species diversity in the rice field, which usually involves people who are not an expert in particular insect groups), identifications of the specimens were carried out by university students who had skills at the taxonomic level of order / family of aquatic insects. Specimens were identified using the available keys and descriptions of the aquatic insects in Thailand (Sangpradub and Boonsoong, 2006; Boonsoong, 2014; Sangpradub, 2016).

DNA extraction, polymerase chain reaction (PCR) and sequencing

Genomic DNA was extracted from larva, nymph or soft tissue part of the adult using the Genomic DNA Extraction mini kit (GF-1 Tissue DNA Extraction Kit, Vivantis, Selangor Darul Ehsan, Malaysia). Polymerase chain reactions (PCR) were conducted for cytochrome c oxidase subunit I (*COI*) using primers LCO1490 (5′-GGTCAACAAATC ATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). PCR reaction and conditions followed the method of Rivera and Currie (2009). Agarose gel electrophoresis

TABLE 1. Sampling locations of the aquatic insects used in this study

Location	Code	Latitude / Longitude	Date
Yang Ta Lad, Kalasin Province	KS1	16° 29' 13" N/103° 12' 42" E	30 Sep 2017
Khao Wong 1, Kalasin Province	KS2	16° 38' 22" N/104° 08' 39" E	07 Oct 2017
Khao Wong 2, Kalasin Province	KS3	16° 32' 09" N/104° 07' 15" E	07 Oct 2017
Yang Sisurat 1, Maha Sarakham Province	MK1	15° 40' 23" N/103° 09' 48" E	28 Oct 2017
Yang Sisurat 2, Maha Sarakham Province	MK2	15° 36' 15" N/103° 09' 41" E	28 Oct 2017
Na Dun, Maha Sarakham Province	MK3	15° 41' 00" N/103° 14' 10" E	28 Oct 2017
Mueang Amnat Charoen, Amnat Charoen Province	AM	15° 49′ 03″ N/104° 37′ 48″ E	4 Nov 2017
Prangku, Si Sa Ket Province	SK	14° 48′ 59″ N/104° 04′ 00″ E	23 Oct 2017

was used to check the PCR products. The HiYield Gel/PCR DNA Extraction Kit (RBC BIOSCIENCE, Taiwan) was used to purified the PCR products and send for sequencing at 1st Base sequencing service (Malaysia) using the same primers as in the PCR.

Data analysis

A total of 98 sequences were included in data analyses. Sequences were deposited in GenBank under accession numbers: MH881228–MH881247 and MH881249–MH881326. To assess the species identification based on *COI* DNA sequence data, the "identification" menu with "Species Level Barcode Records" option in Barcoding of Life Data Systems (http://v4.boldsystems.org) was used. In addition, we also used the Poisson tree processes (PTP) (Zhang et al., 2013) to delimit species of rice field insects and to investigate hidden diversity in morphological species. The maximum likelihood tree was inferred in the RAxML web server version (https://embnet.vital-it.ch/raxml-bb/) (Stamatakis et al., 2008). The output tree was used in PTP species delimitation analyses using the web server version (https://species.h-its.org/) (Zhang et al., 2013). The PTP analysis was run for 100,000 MCMC generations using a thinning value of 100 and 10% burn-in. In addition to the tree-based (i.e. PTP) we also used a distance-based species delimitation method called Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012). ABGD analysis was performed in web server version (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). Criteria setting for ABGD analysis as follow: prior range for maximum intraspecific divergence (0.001, 0.1), minimum slope increase (*X*) of 1.0 and genetic distance calculated based on K80 corrected distances.

To indicate phylogenetic relationships between sequences of the rice field insects included in this study, three methods of phylogenetic analyses were conducted including the neighbor-joining (NJ) tree, the maximum likelihood (ML) and the Bayesian analysis (BA). Because specimens were from distantly related taxa, the phylogenetic trees were analyzed separately for specimens from each order (i.e. Odonata, Ephemeroptera and Hemiptera). The NJ analysis was performed in the MEGA 6 (Tamura et al., 2013) based on Kimura 2-parameter (K2P) model. The branch supports were calculated based on bootstrapping method with 1,000 replications. The ML tree was estimated in PhyML 3.0 (Guindon

et al., 2010). Branch support was calculated using approximate likelihood ratio tests (Anisimova and Gascuel, 2006). Bayesian analysis was performed with MrBayes 3.04b (Huelsenbeck and Ronquist, 2001), and was run for 2,000,000 generations with sampling every of 100 generations and the first 25% were discarded as burn-in. For ML and BA analyses, the jModelTest (Darriba et al., 2012) was used to select the best-fit substitution model for sequences data based on the corrected Akaike information criterion. The COI sequences of *Callibaetis ferrugineus* (GenBank accession number KT707077), *Procladius* sp. and *Tipula* sp. were used as outgroup for phylogenetic analyses of Odonata, Hemiptera and Ephemeroptera, respectively.

RESULTS

Morphological identification

A total of 175 specimens were morphologically examined and 98 were used for molecular analysis (Table 2). Based on morphological characters, only 32 specimens were successfully identified to species level (Table 2). All morphological identifications that could assign a specimen to a species were for the adults of the order Odonata, excepting one adult specimen of Hemiptera that was identified as *Diplonychus rusticus* Fabricius (Table 2). In total, 13 species were recognized. Seven specimens were assigned into genus and the remainder could only be assigned to order or family levels.

DNA barcode species identification and species delimitation

A total of 98 specimens were included in DNA barcode analysis (Table 2). Identification of the specimens based on DNA barcode revealed that 63 of 98 sequences were successfully identified into species level (Table 2). These sequences were belonging to 13 insect species, most (62 sequences) of these were from the order Odonata. For other insect orders (12 specimens of Hemiptera, 3 specimens of Diptera and 5 specimens of Ephemeroptera), only single sequence of the order Diptera was successfully identified in the BOLD (Table 2). The results of DNA barcode and species delimitation of each insect order based on PTP and ABGD methods are as follows.–

Odonata

A total of 78 sequences were obtained and were morphologically identified into 11 species. DNA barcoding in BOLD revealed that 62 specimens were successfully identified into 12 species and 16 specimens were ambiguous identifications (Table 2). The NJ tree (Fig. 1) revealed that all species were formed monophyletic group agree with DNA barcode species identification. The PTP and ABGD species delimitation method revealed that the samples included 17 and 16 species, respectively (Fig. 1).Two specimens of *Pseudagrion australasiae* Sélys were delimited into two species according to the PTP analysis (Fig 1) but ABGD combined them into single species.

Ephemeroptera

Five sequences from the larvae of Ephemeroptera were obtained. These specimens are only morphologically identified to the level of order. DNA barcoding identification of these sequences in BOLD were not successful because no match sequences were found. The NJ tree (Fig. 2) revealed that all five specimens were separated into two groups. Four specimens formed one group and remaining formed another. Both PTP and ABGD species delimitations revealed that these groups represent different species (Fig. 2).

TABLE 2. Specimens used for DNA barcode analysis and the species identification based on morphology and DNA barcoding sequence

Location code	Specimen code	GenBank accession number	Life stage	Morphological identification	DNA barcode identification	Percentage closest match in BOLD
AM	odo1316	MH881259	Larva	Odonata	*Acisoma panorpoides*	99.29
AM	odo1317	MH881260	Larva	Odonata	*Acisoma panorpoides*	99.47
AM	odo1312	MH881271	Larva	Odonata	*Agriocnemis minima*	99.46
AM	odo1319	MH881318	Larva	Odonata	*Crocothemis servilia* *Pantala* sp.	99.13
AM	odo1311	MH881288	Larva	Odonata	*Ischnura senegalensis*	100
AM	odo132	MH881289	Larva	Odonata	*Ischnura senegalensis*	100
AM	odo1321	MH881287	Larva	Odonata	*Ischnura senegalensis*	98.30
AM	odo1325	MH881290	Larva	Odonata	*Ischnura senegalensis*	100
AM	odo1326	MH881291	Larva	Odonata	*Ischnura senegalensis*	100
AM	odo1331	MH881292	Larva	Odonata	*Ischnura senegalensis*	100
AM	odo131	MH881293	Larva	Odonata	*Ischnura senegalensis*	100
AM	odo1329	MH881294	Larva	Odonata	*Ischnura senegalensis*	100
AM	odo1327	MH881295	Larva	Odonata	*Ischnura senegalensis*	99.82
AM	odo135	MH881296	Larva	Odonata	*Ischnura senegalensis*	99.56
AM	odo137	MH881297	Larva	Odonata	*Ischnura senegalensis*	100
AM	odo138	MH881286	Larva	Odonata	*Ischnura senegalensis*	99.15
AM	odo139	MH881298	Larva	Odonata	*Ischnura senegalensis*	99.56
AM	odo1314	MH881326	Larva	Odonata	*Pseudagrion microcephalum*	99.82
AM	odo134	MH881249	Larva	Odonata	*Pseudagrion rubriceps*	99.63
AM	odo136	MH881250	Larva	Odonata	*Pseudagrion rubriceps*	99.63
AM	odo1332	MH881252	Larva	Odonata	*Pseudagrion rubriceps*	98.56
AM	odo1313	MH881254	Larva	Odonata	*Pseudagrion rubriceps*	99.46
AM	odo1330	MH881253	Larva	Odonata	*Pseudagrion rubriceps*	100
AM	odo1320	MH881251	Larva	Odonata	*Pseudagrion rubriceps*	99.28
KS1	2APA1	MH881256	Adult	Odonata, Libellulidae, *Acisoma panorpoides*	*Acisoma panorpoides*	99.11
KS1	odo037	MH881270	Larva	Odonata	*Agriocnemis minima*	98.92
KS1	Cul01	MH881247	Larva	Diptera, Culicidae, *Culex* sp.	*Culex bitaeniorhynchus*	99.11
KS1	Hemi01	MH881242	Adult	Hemiptera	No match	-
KS1	Hele04	MH881243	Adult	Hemiptera, Naucoridae, *Heleocoris* sp.	No match	-
KS1	Noto012	MH881234	Nymph	Hemiptera, Notonectidae, *Nychia* sp.	No match	-
KS1	Noto022	MH881235	Nymph	Hemiptera, Notonectidae, *Nychia* sp.	No match	-
KS1	Noto01	MH881236	Nymph	Hemiptera, Notonectidae, *Nychia* sp.	No match	-
KS1	Noto013	MH881237	Nymph	Hemiptera, Notonectidae, *Nychia* sp.	No match	-
KS1	Tipu03	MH881245	Larva	Diptera, Tipulidae	No match	-
KS2	odo53	MH881302	Larva	Odonata	*Aciagrion pallidum*	100
KS2	odocal62	MH881303	Larva	Odonata	*Aciagrion pallidum*	100
KS2	odo55	MH881281	Larva	Odonata	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	100
KS2	odo52	MH881282	Larva	Odonata	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	98.67
KS2	1CIN1	MH881304	Adult	Odonata, Coenagrionidae *Ceriagrion cerinorubellum*	*Ceriagrion cerinorubellum*	100

TABLE 2. Continue.

Location code	Specimen code	GenBank accession number	Life stage	Morphological identification	DNA barcode identification	Percentage closest match in BOLD
KS2	1CSEM1	MH881316	Adult	Odonata, Libellulidae *Crocothemis servilia*	*Crocothemis servilia* *Pantala* sp.	99.65
KS2	1CSE2FM	MH881317	Adult	Odonata, Libellulidae *Crocothemis servilia*	*Crocothemis servilia* *Pantala* sp.	99.47
KS2	1DNE3	MH881306	Adult	Odonata, Libellulidae, *Diplacodes nebulosa*	*Diplacodes nebulosa*	98.43
KS2	1DNE2	MH881305	Adult	Odonata, Libellulidae, *Diplacodes nebulosa*	*Diplacodes nebulosa*	98.84
KS2	1ISE1	MH881299	Adult	Odonata, Coenagrionidae, *Ischnura senegalensis*	*Ischnura senegalensis*	99.82
KS2	1ISE2	MH881300	Adult	Odonata, Coenagrionidae, *Ischnura senegalensis*	*Ischnura senegalensis*	99.56
KS2	1NTU1	MH881320	Adult	Odonata, Libellulidae, *Neurothemis tullia*	*Neurothemis intermedia*	97.31
KS2	1NTU2	MH881321	Adult	Odonata, Libellulidae, *Neurothemis tullia*	*Neurothemis intermedia*	98.03
KS2	Eph063	MH881229	Larva	Ephemeroptera	No match	-
KS2	Eph62	MH881230	Larva	Ephemeroptera	No match	-
KS2	Eph51	MH881231	Larva	Ephemeroptera	No match	-
KS2	Eph52	MH881232	Larva	Ephemeroptera	No match	-
KS3	1APY3	MH881272	Adult	Odonata, Coenagrionidae, *Agriocnemis minima*	*Agriocnemis minima*	99.04
KS3	odo78	MH881279	Larva	Odonata	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	100
KS3	odo79	MH881280	Larva	Odonata	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	98.67
KS3	odo82	MH881283	Larva	Odonata	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	100
KS3	1COL1	MH881315	Adult	Odonata, Coenagrionidae, *Ceriagrion coromandelianum*	*Ceriagrion coromandelianum*	99.25
KS3	1NTUM2	MH881325	Adult	Odonata, Libellulidae, *Neurothemis tullia*	*Neurothemis tullia*	98.40
KS3	Rus08	MH881244	Adult	Hemiptera, Belostomatidae, *Diplonychus rusticus*	No match	-
KS3	Tipo08	MH881246	Larva	Diptera, Chironomidae	No match	-
KS3	Noto81	MH881233	Nymph	Hemiptera, Notonectidae, *Nychia* sp.	No match	-
KS3	odo83	MH881284	Larva	Odonata	*Pseudagrion australasiae* *Pseudagrion malabaricum*	99.22
KS3	odo84	MH881285	Larva	Odonata	*Pseudagrion australasiae* *Pseudagrion malabaricum*	99.43
MK1	2APA2	MH881257	Adult	Odonata, Libellulidae, *Acisoma panorpoides*	*Acisoma panorpoides*	98.57
MK1	odo91	MH881255	Larva	Odonata	*Acisoma panorpoides*	98.93

TABLE 2. Continue.

Location code	Specimen code	GenBank accession number	Life stage	Morphological identification	DNA barcode identification	Percentage closest match in BOLD
MK1	odo99	MH881261	Larva	Odonata	*Agriocnemis minima*	99.28
MK1	odo910	MH881262	Larva	Odonata	*Agriocnemis minima*	98.91
MK1	odo92	MH881263	Larva	Odonata	*Agriocnemis minima*	99.09
MK1	odo93	MH881264	Larva	Odonata	*Agriocnemis minima*	98.01
MK1	odo94	MH881265	Larva	Odonata	*Agriocnemis minima*	99.82
MK1	odo95	MH881266	Larva	Odonata	*Agriocnemis minima*	99.64
MK1	odo96	MH881267	Larva	Odonata	*Agriocnemis minima*	99.64
MK1	odo97	MH881268	Larva	Odonata	*Agriocnemis minima*	99.82
MK1	odo98	MH881269	Larva	Odonata	*Agriocnemis minima*	99.46
MK1	3APY11	MH881273	Adult	Odonata, Coenagrionidae, *Agriocnemis pygmaea*	*Agriocnemis pygmaea*	100
MK1	2DTR2	MH881312	Adult	Odonata, Libellulidae, *Diplacodes trivialis*	*Diplacodes trivialis*	100
MK1	Eph91	MH881228	Larva	Ephemeroptera	No match	-
MK2	3APY1	MH881276	Adult	Odonata, Coenagrionidae, *Agriocnemis pygmaea*	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	100
MK2	3DNE3	MH881307	Adult	Odonata, Libellulidae, *Diplacodes nebulosa*	*Diplacodes nebulosa*	98.64
MK2	3DTR1	MH881308	Adult	Odonata, Libellulidae, *Diplacodes trivialis*	*Diplacodes trivialis*	99.22
MK2	3DTR2	MH881309	Adult	Odonata, Libellulidae, *Diplacodes trivialis*	*Diplacodes trivialis*	99.61
MK2	3DTR3	MH881310	Adult	Odonata, Libellulidae, *Diplacodes trivialis*	*Diplacodes trivialis*	99.03
MK2	3DTR4	MH881311	Adult	Odonata, Libellulidae, *Diplacodes trivialis*	*Diplacodes trivialis*	99.40
MK2	2DTR1	MH881314	Adult	Odonata, Libellulidae, *Diplacodes trivialis*	*Diplacodes trivialis*	99.21
MK2	GPR	MH881319	Adult	Odonata, Libellulidae, *Orthetrum pruinosum*	*Orthetrum pruinosum* *Orthetrum pruinosum neglectum* *Orthetrum testaceum*	99.79
MK3	odo113	MH881277	Larva	Odonata	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	99.81
MK3	odo112	MH881278	Larva	Odonata	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	99.81
MK3	odo114	MH881301	Larva	Odonata	*Ischnura senegalensis*	99.64
MK3	Hemi111	MH881238	Adult	Hemiptera	No match	-
MK3	Hemi112	MH881239	Adult	Hemiptera	No match	-
MK3	Hemi113	MH881240	Adult	Hemiptera	No match	-
MK3	Hemi114	MH881241	Adult	Hemiptera	No match	-
SK	4APA1	MH881258	Adult	Odonata, Libellulidae, *Acisoma panorpoides*	*Acisoma panorpoides*	99.11
SK	4APY1	MH881274	Adult	Odonata, Coenagrionidae, *Agriocnemis pygmaea*	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	99.42
SK	4APY2	MH881275	Adult	Odonata, Coenagrionidae, *Agriocnemis pygmaea*	*Agriocnemis pygmaea* *Ischnura senegalensis* *Agriocnemis femina*	99.81

TABLE 2. Continue.

Location code	Specimen code	GenBank accession number	Life stage	Morphological identification	DNA barcode identification	Percentage closest match in BOLD
SK	4DTR1	MH881313	Adult	Odonata, Libellulidae, *Diplacodes trivialis*	*Diplacodes trivialis*	99.40
SK	4NTU21	MH881322	Adult	Odonata, Libellulidae, *Neurothemis tullia*	*Neurothemis tullia*	98.57
SK	4NTU3	MH881323	Adult	Odonata, Libellulidae, *Neurothemis tullia*	*Neurothemis tullia*	98.04
SK	4NTU31	MH881324	Adult	Odonata, Libellulidae, *Neurothemis tullia*	*Neurothemis tullia*	98.22

Details of location code are provided in Table 1.

Hemiptera

Twelve sequences were obtained from the order Hemiptera. Morphological identifications revealed that they are comprised of *D. rusticus* (n=1), genus *Nychia* Stål (n=5) and *Heleocoris* Stål (n=1) and remaining five specimens were only identified at the order level (Table 2). Like those of the Ephemeroptera, DNA barcode identification in BOLD found no match for sequences obtained from this order. NJ tree (Fig. 3) revealed three groups, all with strong supported. These groups comprised of four species based on PTP species delimitation analysis. However, the ABGD method delimits specimens of the order Hemiptera into three species (Fig. 3). All five sequences from the specimens that were morphologically identified at the order level formed one species. The genus *Nychia* and genus *Heleocortis* formed two separated species with *D. rusticus* forming another (Fig. 3).

Diptera

Only three sequences were obtained from the order Diptera. Two specimens were morphologically identified into genus *Tipula* Linnaeus of the family Tipulidae and one specimen was identified into the genus *Culex* Linnaeus of the family Culicidae (Table 2). DNA barcode analysis in BOLD found that the *Culex* specimen was match with *Culex bitaeniorhychus* Giles with high percent similarity (99.11%). However, two sequences of the genus *Tipula* found no match in BOLD. The PTP species delimitation revealed two but the ABGD method found one species of Diptera. Two sequences of the genus *Tipula* belonged to the same species and the *Culex* specimen formed another species.

DISCUSSION

Aquatic insects play diverse roles in the rice field ecosystem. Therefore, knowledge of taxonomic and species diversity is crucial for all aspects of further study of these organisms. We have revealed that mitochondrial *COI* sequences could be successfully used for species identification of one of the major groups of rice field insects, the order Odonata. The major advantage of DNA barcoding is that it can be used to associate different life stages (Miller et al., 2005; Gattolliat and Monaghan, 2010; Pramual and Wongpakam, 2014).

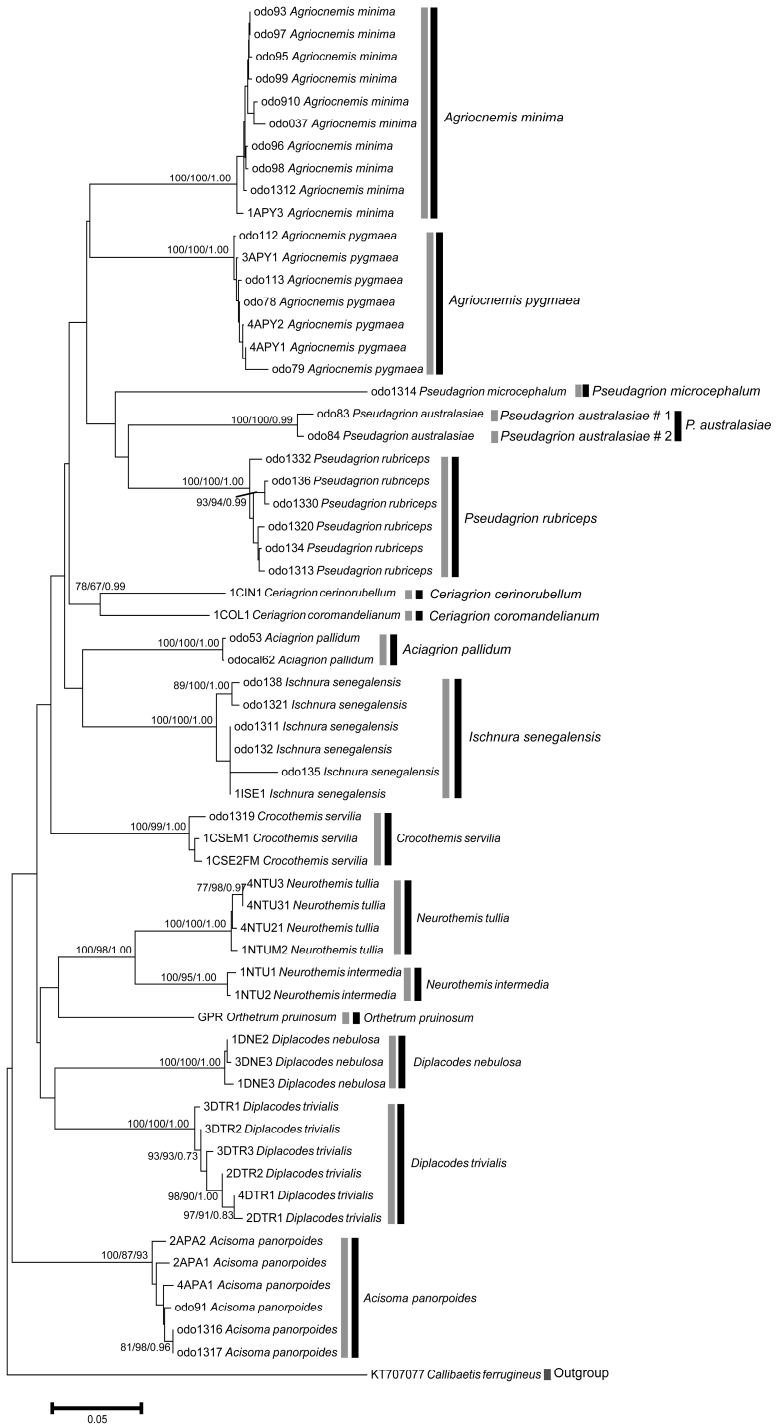

FIGURE 1. Neighbor-joining tree for 61 haplotypes of the mitochondrial cytochrome c oxidase I (COI) sequences of Odonata collected from rice fields, Thailand. Bootstrap values for neighbor-joining and maximum likelihood (ML) and posterior probability of Bayesian analysis are shown above or near the branches. Scale bar represents 0.05 substitutions per nucleotide position. Vertical bar indicates species delimited by Poisson tree processes (PTP) (grey) and Automatic Barcode Gap Discovery (ABGD) (black) methods.

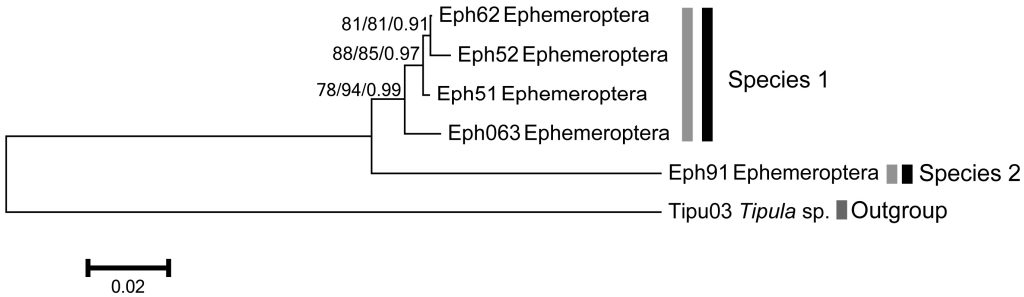

FIGURE 2. Neighbor-joining tree for 5 sequences of the mitochondrial cytochrome c oxidase I (COI) sequences of 4 taxa of Ephemeroptera from Thailand. Bootstrap values for neighbor-joining and maximum likelihood (ML) and posterior probability of Bayesian analysis are shown above or near the branches. Scale bar represents 0.05 substitutions per nucleotide position. Vertical bar indicates species delimited by Poisson tree processes (PTP) (grey) and Automatic Barcode Gap Discovery (ABGD) (black) methods.

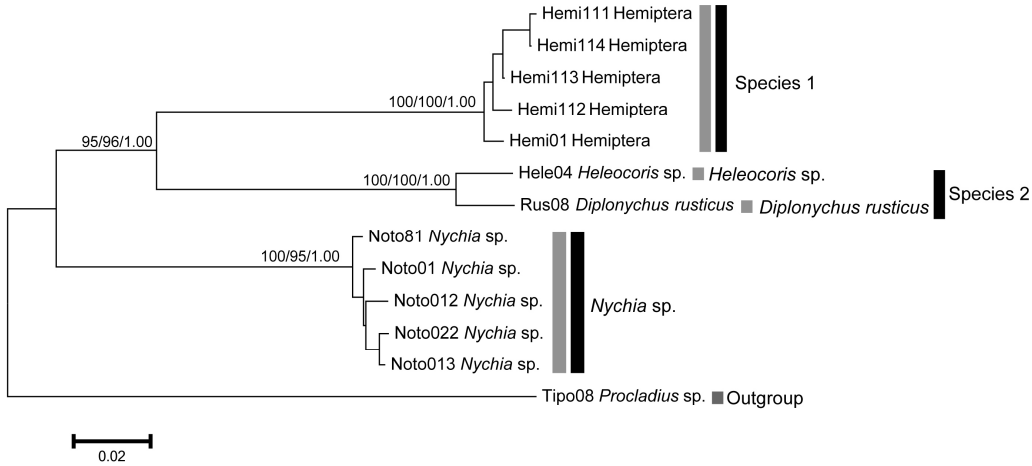

FIGURE 3. Neighbor-joining tree for 12 sequences of the mitochondrial cytochrome c oxidase I (COI) sequences of two taxa of Hemiptera from Thailand. Bootstrap values for neighbor-joining and maximum likelihood (ML) and posterior probability of Bayesian analysis are shown above or near the branches. Scale bar represents 0.05 substitutions per nucleotide position. Vertical bar indicates species delimited by Poisson tree processes (PTP) (grey) and Automatic Barcode Gap Discovery (ABGD) (black) methods.

This is highly useful for the insects which are taxonomically challenging in some life stages. For example, identification of the larva of Odonata based on morphology is very difficult and all of our specimens could only be assigned to the level of order. In contrast, DNA barcodes can associate 33 larvae with three conspecific adults. Although the remaining (9) larvae were not associated with our adult sequences, they could be successfully identified to species in DNA barcode database (i.e. BOLD).

Despite a considerable rate (80%, 62 sequences) of successful identification for

Odonata in BOLD, some (20%, 16 sequences) were ambiguous and raised the problem of the uncertainty of the taxonomic identity of reference sequences in the public database (Pramual et al., 2016). These sequences are matching with more than one species in the database. It could be possible that some of these species (*Agriocnemis femina* Brauer and *A. pygmaea* Rambur; *P. australasiae* and *P. malabaricum* Fraser) are closely related species (O'Grady and May, 2003) thus, lineage sorting might not yet be complete (Funk and Omland, 2003) resulting in ambiguous identification of these specimens. Closely related species are often problematic for DNA barcoding of insects (Elias et al., 2007, Pramual and Adler, 2014) including members of the Odonata (Rach et al., 2008). Another possibility is that the sources of some sequences deposited in the public database (e.g. BOLD and GenBank) might be incorrectly identified. For example, our specimens of *Crocothemis servilia* Drury were match with *C. servilia* and *Pantala* sp. in BOLD. These two genera could clearly be differentiated based on morphological characters. Thus, ambiguous identification of these specimens is most likely due to erroneous identification of the sources of sequences deposited in the public database. This issue has already been raised by several studies (e.g. Nilsson et al., 2006, Shen et al., 2013; Pramual et al., 2016) and suggests some limitations of DNA barcoding.

Specimens from other orders including Diptera, Ephemeroptera and Hemiptera were far less successfully identified in BOLD; just one sequence from Diptera (*C. bitaeniorhychus*) was successfully identified. This is because of a lack of reference sequences in the database. One possible way of documenting biodiversity if there are no reference sequences is to use the DNA-based, species delimitation method. The PTP and ABGD analyses revealed 25 and 23 species, respectively, among 98 sequences included in the analysis. There is one more species from the Coleoptera included in this study but PTP analysis could not be performed because only a single specimen was included in this study. Thus, in total we found 26 of the aquatic insect species in the rice field among 99 specimens. Although this number of species is far less than the possible diversity of insects in the rice field ecosystem, it was more than twice that based on morphological identification (13 species). This indicates that DNA barcoding sequences are potentially useful for documenting biodiversity in this under investigated ecosystem in Thailand.

In conclusion, we found that DNA barcode sequences are successful for identification of Odonata in the rice field as several species have reference sequences in the public database (e.g. BOLD). However, the approach had far less success with other insect orders because of limitations of the reference sequence library. Therefore, this should be a priority topic for study in the future. When the species are unknown, we have demonstrated in this study that numbers of species can still be documented using the molecular species delimiting approach. Thus, despite lacking names for species, DNA barcode can be potentially used for biodiversity assessment.

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