

## NGS-barcodes, haplotype networks combined to external morphology help to identify new species in the mangrove genus *Ngirhaphium* Evenhuis & Grootaert, 2002 (Diptera: Dolichopodidae: Rhaphiinae) in Southeast Asia

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**Abstract.** A review is given of the *Ngirhaphium* species of Southeast Asia and two new species are described from Thailand. *Ngirhaphium meieri* Samoh & Grootaert, new species is described from a mangrove in Takua Pa district, Phang Nga Province, peninsular Thailand. NGS-barcodes (313 bp) are congruent with traditional taxonomic delimitation. *Ngirhaphium caeruleum* Grootaert & Puniamoorthy sensu lato seems to consist of a species-complex with 32 haplotypes and has a wide distribution in the southern part of the South China Sea. There is a 4.2 % genetic difference between the Thai/Cambodian population and the Singapore/Brunei populations in *N. caeruleum*: despite the many haplotypes and differences in male terminalia, the Thai and Cambodian haplotypes formed a haplotype group, while the Singapore and Bruneian specimens formed another haplotype group. Nonetheless, the Thai population is morphological distinct enough to be considered a morpho-species: it is here described as *N. thaicum* Samoh & Grootaert, new species. An updated distribution of the six Southeast Asian *Ngirhaphium* species is given with additional taxonomic notes, a key to the species and a phylogenetic diagram. We find that the Andaman Sea coast to be the most diverse with four species while the South China Sea region has only two species both belonging to the *caeruleum* species complex. *N. sivasothii* Grootaert & Puniamoorthy is common and occurring from Singapore up to the Thai coast of the Andaman Sea, with 28 haplotypes. Remarkable in all species is the limited distribution of the haplotypes, where most seem endemic to a limited area and thus provide information about the origin of the distribution.

**Key words.** Dolichopodidae, *Ngirhaphium*, NGS-barcodes, peninsular Thailand

### INTRODUCTION

Peninsular Thailand, located in the southern part of Thailand, is considered a biodiversity hotspot in Southeast Asia and assumed to be a natural corridor or filtration for native and non-native fauna and flora distribution in the region. Patterns of flora distribution and species diversity show a strong separation between lower and upper part of Isthmus of Kra (Whitmore, 1984, 1998; Baker et al., 1998; Wikramanayake et al., 2002), while the faunal distribution of vertebrates e.g., amphibians (Inger, 1999), forest birds (Hughes et al., 2003) and bats (Hughes et al., 2011) have been extensively documented. Unfortunately, few such studies have been conducted on insect diversity and distribution, and are often neglected in analyses.

Although the *Ngirhaphium* Evenhuis & Grootaert, 2002 consists of quite large flies, it remained unnoticed for a long time and the genus was only recently described with *N. murphyi* Evenhuis & Grootaert, 2002 as type species found in Singapore. Two more species, *N. caeruleum* Grootaert & Puniamoorthy, 2014 and *N. sivasothii* Grootaert & Puniamoorthy, 2014 were further found and described after an extensive survey of the mangroves of Singapore. A survey of the mangroves of southern Thailand revealed the presence of another new species along the coast of the Andaman Sea: *N. chutamasae* Samoh, Boonrotpongi & Grootaert, 2015. Recently, yet another new species was recorded and is described here as *N. meieri*, new species.

All the *Ngirhaphium* species were barcoded with Next Generation Sequencing techniques described in Meier et al. (2016) and Wang et al. (2018). This way we obtained more than 2,200 NGS barcodes (313 bp) for all the species actually known in Southeast Asia. This quick and cheap barcode technique is now currently used (Ang et al., 2013; Ang et al., 2017; Ramos et al., 2018) allowing the detection of cryptic species as well as the matching of females to males (Yeo et al., 2018). Here we illustrate with the case of the *N. caeruleum* species complex presenting a case of cryptic species. There is a 4.2 % genetic difference between

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the Thai population and the Singapore/Brunei populations in *N. caeruleum*. As will be shown, a relation was found between the different haplotypes and the male terminalia of specimens from Thailand and Cambodia on one hand and the specimens from Singapore and Brunei on the other hand. This leads us to describe the Thai/Cambodian population as new to science.

In building a haplotype network (Clement et al., 2000; Santos et al., 2015) for the respective species, we were able to analyse the genealogy and patterns of distribution of the haplotypes in Southeast Asia.

To summarise, in the present paper we provide a key and diagnoses for the six species actually known from Southeast Asia and we describe two new species for science. Haplotype networks are analysed and discussed.

## MATERIAL & METHODS

**Specimen collection and preservation.** The present study is primarily based on an intensive survey of mangroves in southern and eastern Thailand, Cambodia, Singapore, and Brunei.

The survey in Thailand was done by the first author by using Malaise traps, sweep netting and yellow pan traps. All fresh specimens were preserved in 70% ethyl alcohol and deposited in the collections of the Princess Maha Chakri Sirindhorn Natural History Museum (PSUNHM) of the Prince of Songkla University (PSU), Hat Yai, Songkhla, Thailand. In addition, voucher specimens are also preserved in the collections of the Royal Belgian Institute of Natural Sciences (RBINS), Brussels, Belgium.

The surveys in Singapore (Grootaert, 2018), Brunei (collected Claas Damken) and Cambodia (collected Jérôme Constant) were done exclusively with Malaise traps and the material is conserved in the Lee Kong Chian Natural History Museum (NUS, Singapore) with vouchers in the Royal Belgian Institute of Natural Sciences (RBINS), Brussels, Belgium.

**External morphology analysis.** Adult male and female flies were measured using Olympus SZ Stereo microscope for wing and body length. Male genitalia were drawn with a Leica DM 2000 compound microscope equipped with a Camera Lucida (drawing tube-Leica Microsystem Deerfield, IL). The left lateral side of the male hypopygium was illustrated, inked, and enhanced using Adobe Photoshop CS6. Long-legged fly morphological terminology follows Grootaert & Puniamorthy (2014).

All measurements are shown in millimetres and were made on representative wet specimens.

**Photography.** A focus stacking technique (see Brecko et al., 2014) was used to image the specimens. The high resolution pictures were stacked using Zerene Stacker software. The scale on the photos is 1 mm.

**Taxon names.** The new taxon names *Ngirhaphium thaicum* Samoh & Grootaert, new species and *Ngirhaphium meieri* Samoh & Grootaert, new species are the responsibility of Abdulloh Samoh and Patrick Grootaert. The authorship of the new taxon name should be cited as *Ngirhaphium thaicum* Samoh & Grootaert in Samoh, Satasook & Grootaert, 2019 and *Ngirhaphium meieri* Samoh & Grootaert in Samoh, Satasook & Grootaert, 2019.

## MOLECULAR ANALYSIS

**PCR amplification and Next Generation Sequencing (NGS) barcoding.** NGS barcodes were amplified from parts of legs using the direct polymerase chain reaction (direct PCR) protocol following Wong et al. (2014) in order to avoid DNA extraction. The leg tissues were immersed in prepared mixtures of 20 µl volume each (2 µl of 10× BioReady rTaq 10× Reaction Buffer, 1.5 µl of 2.5 mM dNTP mix, 0.2 µl of BioReady rTaq DNA polymerase, 2 µl each of 5 µM forward and reverse primers, 2 µl of 1mg/ml Bovine Serum Albumin, RNase/DNase-free sterile water) and otherwise processed following the manufacturer's instructions. Thermocycling conditions were processed as follow: initial denaturation at 95°C for 3 mins, followed by 40 cycles of denaturation at 94°C for 1 min., annealing at 48°C for 1 min., and extension at 72°C for 1.5 mins. A final extension of 5 mins at 72°C was used. Gel electrophoresis was performed to verify the PCR success in a 1% agarose gel using 5 µl of the reaction mix. All PCR products were combined and cleaned in aliquots of up to 100 µl using SureClean (Bioline Inc., London, UK); cleaned amplicon products were re-eluted in RNase/DNase-free water. Moreover, amplified PCR products were pooled and sent for further library preparation. NGS libraries were prepared with the combined PCR products by AITbiotech, using the TruSeq Nano DNA Library Preparation kit (Illumina) and then sequenced on an Illumina MiSeq 2×300 bp platform. Paired-end (PE) read data (.fastq) were merged using PEAR ver.0.9.6 (Zhang et al., 2014).

**Phylogeny.** Estimates of evolutionary divergence between sequences were conducted in MEGA7 (Tamura et al., 2004; Kumar et al., 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-1002.1064) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 313 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

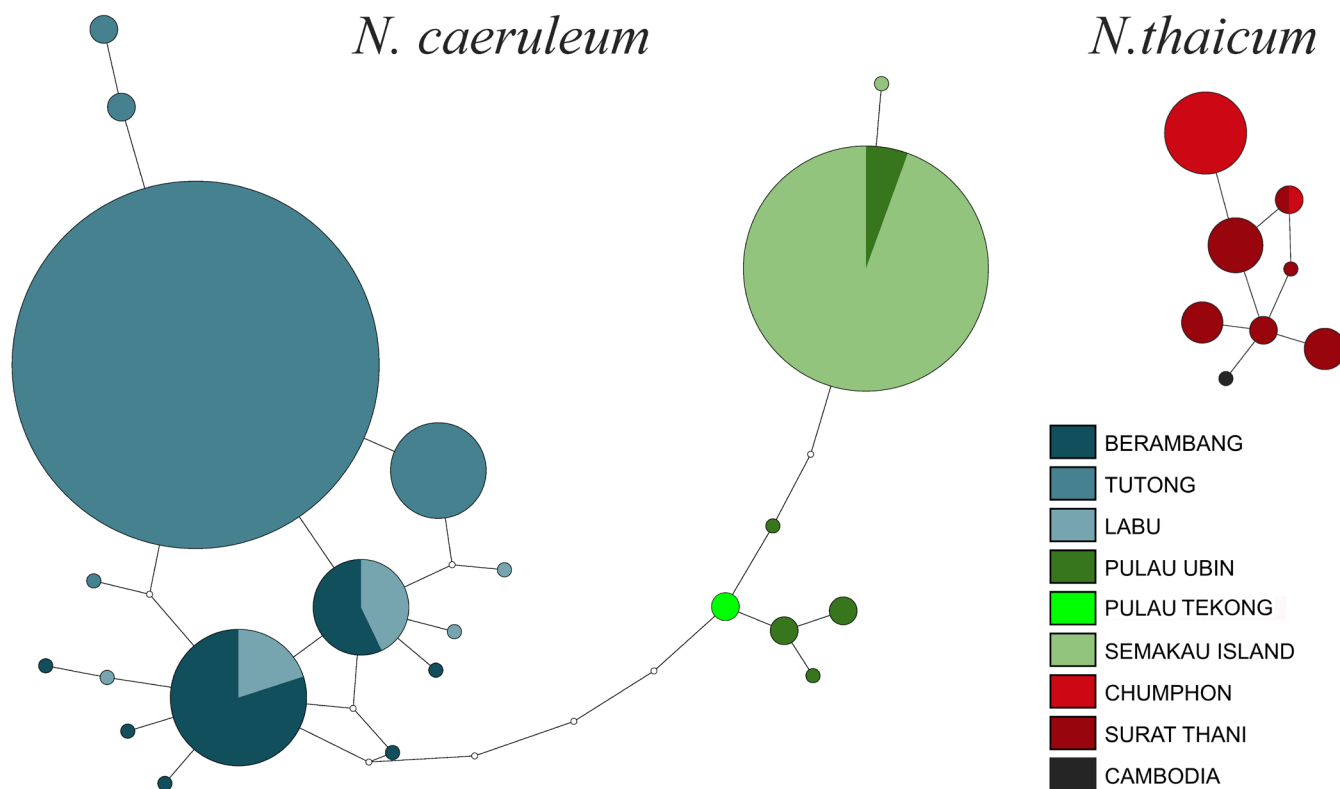


Fig. 1. Haplotype network of the *Ngirhaphium caeruleum*-complex. Brunei: Berambang, Tutong, Labu; Singapore: Pulau Ubin, Pulau Tekong, Semakau Island; Thailand: Chumphon, Surat Thani, and Cambodia.

**Haplotype network and gene genealogy analyses.** In this study, we adopted TCS 1.21 software (Clement et al., 2000) and tcsBU (Santos et al., 2015) to build and illustrate a statistical parsimony haplotype network of selected sequences from both sides of the Thai seacoasts and including some sequences from Singapore, Cambodia, and Brunei.

## RESULTS

### TAXONOMY

#### Family Dolichopodidae

#### Subfamily Rhaphiinae

#### Genus *Ngirhaphium* Evenhuis & Grootaert

*Ngirhaphium* Evenhuis & Grootaert, 2002: 310. Type species by original designation: *Ngirhaphium murphyi* Evenhuis & Grootaert, 2002.

**Diagnosis.** Medium to large sized species (4.5–8 mm) with a metallic green or blue ground-colour. Antenna very long in males, a little shorter in females. Arista apical, basal segment long. Rostrum in male small with well-developed labellae, being much larger in female. Vertex excavated (cf. Sciapodinae). Mid and hind coxae without exterior bristle. Femora with inconspicuous bristling. All tibiae with strong bristles. Fore leg in male with tarsomere 4 bearing an asymmetrical, apical dorsal forked protuberance (absent

in females); apical tarsomere with a pair of normal claws and a thickened claw-like structure beneath the posterior claw. Females with unmodified fore tarsomeres except for the terminal tarsomere that bears a long dorsal apical protuberance. Mid and hind legs with tarsomeres 1–4 with an apical comb of spinules ventrally. Wing with tip of  $M_{1+2}$  sharply bent upwards just before reaching the wing border and ending near tip of  $R_{4+5}$ .

#### *Ngirhaphium caeruleum* species complex

The original described population of *N. caeruleum* consisted of large flies with a blue shine on the thorax and the abdomen and they were accordingly named *caeruleum*, which means blue in Latin. Actually a large population was found on the island of Semakau (Fig. 1). However green metallic shining specimens of *N. caeruleum* were found in southern Thailand, Brunei, Cambodia and on some small islands around Singapore. Apart from their green body colour, their morphology and the male terminalia resemble the original population from Semakau Island in Singapore, but differ in details. Here we paid attention to the shape and the bristling of the surstyli that are clasping organs. We compared it with the haplotypes based on the NGS barcodes (313 bp).

Fig. 2D represents the original blue population from the type locality on Semakau. On the inside of both right and left surstyli there are small bristles, that are black on the inside but pale on the border of the plate. A few small protuberances are also visible on the border of the plates.

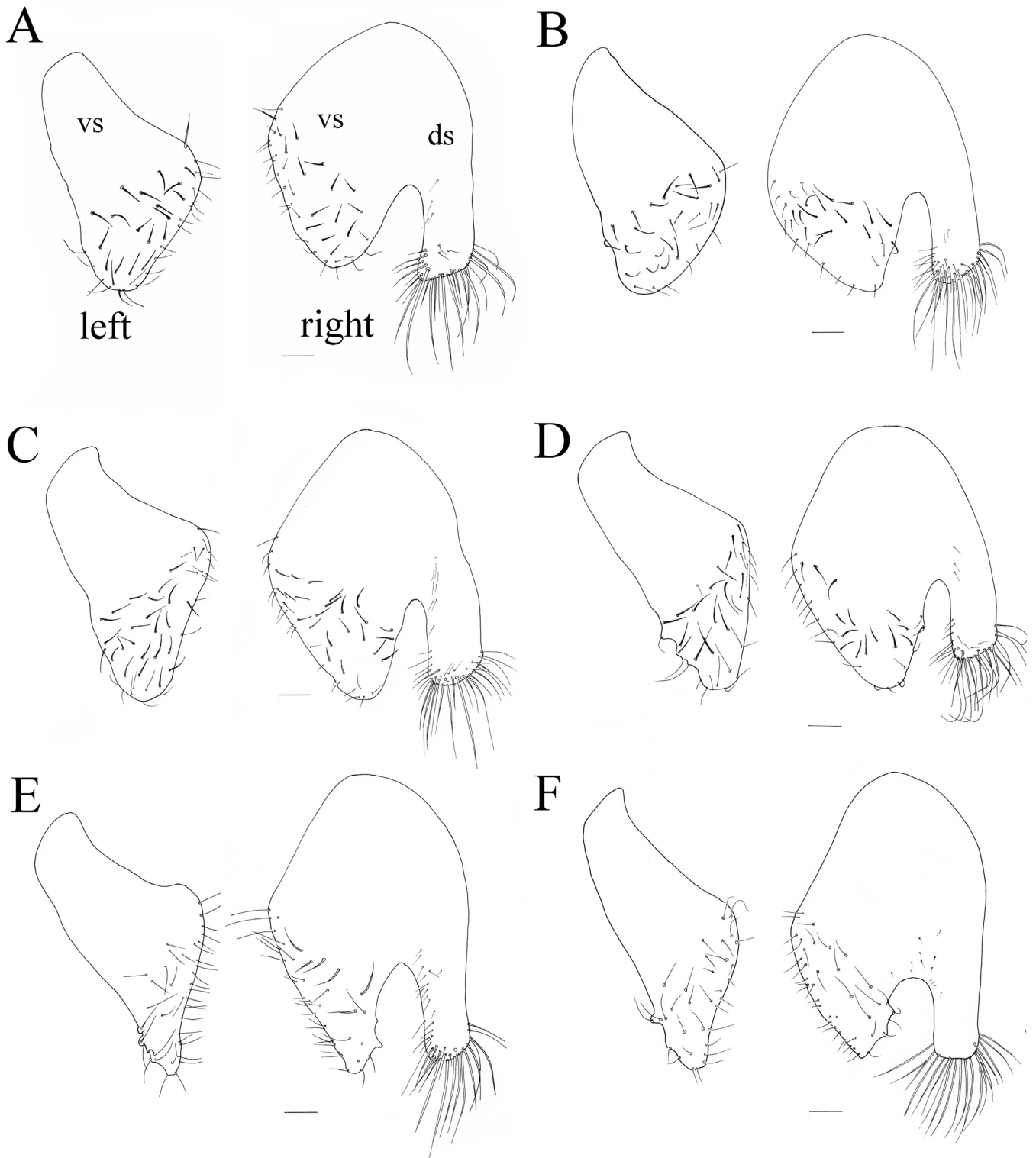


Fig. 2. *Ngirhaphium caeruleum*-complex. View of inside of the dorsal surstylus of the left side and the fused dorsal and ventral surstyli of the right side. Scale = 0.1mm. A, green *caeruleum* from Tutong, Brunei (ZRCBDP0066395) at 1.6 % from the Semakau population; B, green *caeruleum* from Pulau Tekong, Singapore (ZRCBDP0001462) at 0.6% from the Semakau population; C, green *caeruleum* from Pulau Ubin, Singapore (ZRC\_BDP\_0084430) at 0.6% from the Semakau population; D, blue *caeruleum* from the type locality on Semakau Island, Singapore (ZRCBDP0118762); E, green *thaicum*, new species from Surat Thani, Thailand (24-018) at 4.2% from the Semakau population; F, green *thaicum*, new species from Cambodia (JP3C\_Ngi-cambodiensis\_Misc002) within the variability of the southern Thailand populations.

The populations from Brunei (localities Tutong, Berambang, and Labu, Fig. 1) have the inside of the surstyli and the border covered with small black bristles without any tubercles. Both surstyli are comparatively wide and the tips are bluntly rounded (Fig. 2A). The populations on Pulau Tekong and Pulau Ubin (Fig. 2B, C) have similar shaped surstyli as in Brunei with short black bristles.

In the Thai and Cambodian population both surstyli are distinctly more pointed (Fig. 2E, F) and the tubercles on the border and sometimes on the inside of the surstyli are more pronounced. The inside of the plates are covered with short yellow bristles.

This distinct morphology and since the genetic distance of the Thai/Cambodian population is 4.2% apart from the Singapore and Brunei population let us decide to consider the Thai and Cambodian population as a different species. Considering it just as a variable species would only lead to loss of information linked to its ecology. The new species is described here below. If we consider the bio-species concept it might well be that the blue *N. caeruleum* from Semakau represents a distinct bio-species from the green specimens from the surrounding islands of Singapore and the populations on Brunei. However, the short genetic distance of 1–2% difference and the similar morphology except for the general body colour would render this species unrecognisable.

**Haplotype network.** Number of sequences used: 114

Total number of sites (excluding sites with gaps / missing data): 311

Number of haplotypes, h: 31.

There are seven haplotypes of the *caeruleum* complex in Thailand and one in Cambodia. They are all unique as can be seen on Fig. 1. There is no connection shown on the figure between the Thai and the Singaporean population because the genetic distance (4.2%) is too far. The original blue *caeruleum* population is found on the island of Semakau. On Semakau Island there are only 2 haplotypes: 1 huge population and a very small one. The Semakau population is connected to the Pulau Ubin population where 5 haplotypes occur. One haplotype is shared with Semakau. Pulau Tekong, which is very close to Pulau Ubin has an own unique haplotype. This population seems to be connected to the populations on Brunei. Remarkable is that the connection is by haplotypes occurring in the bay of Brunei (Berambang and Labu) and not the population in Tutong that is closer to Singapore. That population is well separated from the 2 sites in the Bay of Brunei.

***Ngirhaphium caeruleum* Grootaert & Puniamoorthy, 2014**  
(Fig. 2D, 10C)

*Ngirhaphium caeruleum* Grootaert & Puniamoorthy, 2014: 154 (figs. 11, 12, 13–16, 19). Type locality Semakau.

**Diagnosis.** A larger species with mesonotum and tergites shining metallic blue. Apical arisal segment thin, slightly longer than basal arisal segment. Male with dorsal and ventral surstylus fused at the right side, separated at the left side. Cerci long, but shorter than the surstyli so that they are concealed between the surstyli.

**Material examined.** Singapore: Semakau Island: 253 specimens (103 specimens NGS barcoded, LKCNHM)

Brunei: Tutong, Berambang, and Labu more than 800 specimens (476 specimens barcoded, LKCNHM).

**Distribution.** Singapore and Brunei.

***Ngirhaphium thaicum* Samoh & Grootaert, new species**  
(Figs. 2E, F, 3–5)

**Material examined.** Holotype male: THAILAND: Ban Nua Nam, Phum Rieng, Chaiya, Surat Thani (9°23'34.0"N, 99°15'24.0"E), Samoh, A., sweep netting, 22 April 2015 (PSUNHM). (barcode: Thai\_27-018\_Abdullah\_ABDO08\_SuratThani)

Paratypes: THAILAND: 1 male, Ban Elet, Tha Yang, Mueang, Chumphon province (10°22'24.0"N 99°13'35.4"E), Samoh, A., sweep netting, 16 February 2015. (barcode: Thai\_27-004\_Abdullah\_ABDO07\_Chumphon). 1 male, Ban Nua Nam, Phum Rieng, Chaiya, Surat Thani (9°23'34.0"N, 99°15'24.0"E), Samoh, A., sweep netting, 18 April 2015. (barcode: Thai\_27-009\_Abdullah\_ABDO07\_SuratThani). 1 female, Ban Elet, Tha Yang, Mueang, Chumphon province (10°22'24.0"N 99°13'35.4"E), Samoh, A., sweep netting, 16 February 2015. (barcode: Thai\_27-006\_Abdullah\_ABDO07\_Chumphon). 4 males; 10 females, Ban Elet, Tha Yang, Mueang, Chumphon province (10°22'24.0"N 99°13'35.4"E), Samoh, A., sweep netting, 16 February 2015. 1 male; 1 female, Ban Elet, Tha Yang, Mueang, Chumphon province (10°22'24.0"N 99°13'35.4"E), Samoh, A., Malaise trap, 18 February 2015. 1 female, Khao Than, Tha Chang, Surat Thani province (9°19'43.4"N, 99°12'31.6"E) Samoh, A., Malaise trap, 21 April 2015. 1 male; 1 female, Ban Nua Nam, Phum Rieng, Chaiya, Surat Thani (9°23'34.0"N, 99°15'24.0"E), Samoh, A., Malaise trap, 22 April 2015. 8 males; 12 females, Ban Nua Nam, Phum Rieng, Chaiya, Surat Thani (9°23'34.0"N, 99°15'24.0"E), Samoh, A., sweep netting, 18 April 2015. 3 males, 6 females, Khlong Wan, Muang, Prachuap Kirikhan (11°45'26.2"N 99°47'36.3"E), 8 July 2018 Samoh, A. 2 males; 1 female, Ban Salak Khok, Koh Chang, Trat (12°01'30.1"N 102°23'35.9"E), Samoh, A., sweep netting, 3 November 2018. 2 female, Pak Nam, Sawee, Chumphon province (10°16'41.5"N 99°09'43.9"E), Samoh, A., sweep netting, 7 July 2018 (PSUNHM). CAMBODIA: 1 male, 2 females, Koh Kong province, Peam Krasaop WS, Boeng Kayak 11°30'31"N 103°05'50"E 9–10 October 2015 (Malaise trap, collected J. Constant & J Bresseel; RBINS). Male with barcode reference JP3C\_Ngi-cambo\_Misc002.



Fig. 3. *Ngirhaphium thaicum*, new species male habitus. Scale = 1 mm.

**Extended diagnosis.** Male (Fig. 3) Body: 7.2–7.7 mm; wing: 5.6–5.9 mm. A large species of the *caeruleum* complex with mesonotum and tergites shining metallic green. Antenna: scape: 0.39; pedicel: 0.12; postpedicel: 0.82; basal arisal segment: 0.2; apical arisal segment: 0.325 (all in mm). Apical arisal segment thin (filiform), about 1.5 times as long as basal arisal segment.

Thorax with a green metallic shine.

Wing (Fig. 3) clear, but anterior two cells brownish tinged. Veins black and weakly seamed brown.

Legs (Fig. 3). Fore leg. Tarsomere 4 with an apical fork, the inner prong (anterior) much longer than the outer (posterior) prong. Tarsomere 5 with an apical protuberance. Ratio of femur, tibia and tarsomeres in mm: 1.4 : 1.2 : 0.9 : 0.2 : 0.3 : 0.16 : 0.2.



Fig. 4. *Ngirhaphium thaicum*, new species habitus female. Scale = 1 mm.

Mid leg. Tarsomere 5 with some longer anterior and posterior bristles though not forming a flag. Ratio of femur, tibia and tarsomeres in mm: 1.96 : 2.6 : 1.36 : 0.64 : 0.54 : 0.28 : 0.28.

Hind leg: Ratio of femur, tibia and tarsomeres in mm: 1.92 : 3.2 : 1.4 : 0.68 : 0.54 : 0.32 : 0.26.

Abdomen with a green metallic shine.

Male terminalia (Fig. 5) with dorsal and ventral surstylus at right side fused (Fig. 2E, 5C); dorsal and ventral surstyli separated at left side. Tip of both right and left dorsal surstyli pointed; tip on inside with short yellow bristles. Right and left ventral surstylus with dorsal border and sometimes the inside bearing a few protuberances. Cerci a little shorter than surstyli so that they are concealed between the surstyli.

Female (Fig. 4) Body: 7.7–8 mm; wing: 6.7–6.9 mm. Similar to male but lacking the apical fork on the fore tarsomere 4 as well as the prong under the anterior claw.

**Distribution.** Trat, Prachuap Kirikan, Chumphon, Surat Thani Province, and Cambodia. Gulf of Thailand (South China Sea).

**Remarks.** This is one of the largest *Ngirhaphium* species. They prefer open areas without canopy cover and with soft mudflat.

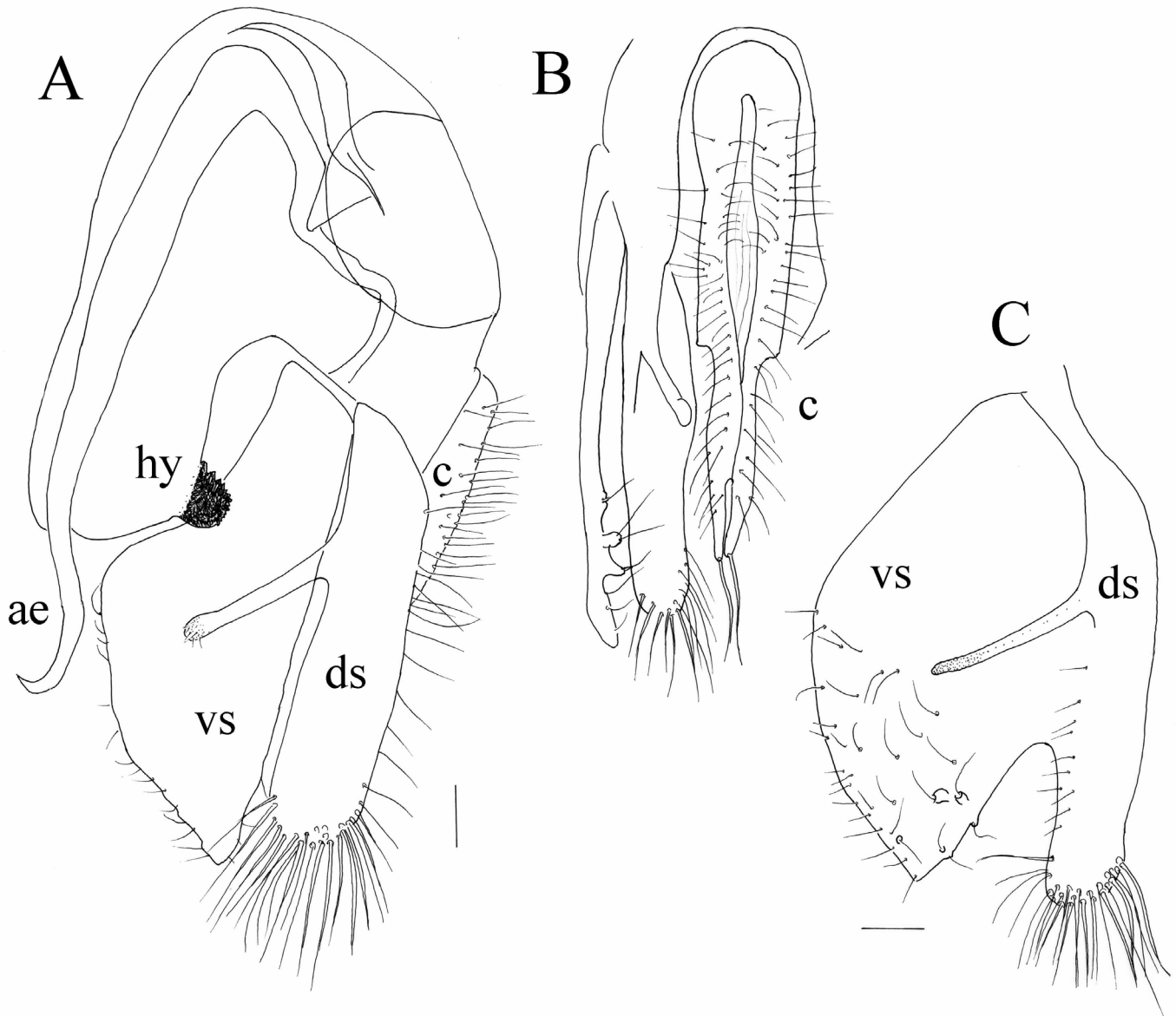


Fig. 5. *Ngirhaphium thaicum*, new species male terminalia (27\_009) A, epandrium left side; B, cerci dorsal view; C, left surstylus inside view. Scale = 0.1 mm.

***Ngirhaphium chutamasae* Samoh, Boonrotpong & Grootaert, 2015**  
(Fig. 10E)

*Ngirhaphium chutamasae* Samoh, Boonrotpongi & Grootaert, 2015: 126 (figs. 1–6). Type locality THAILAND Tammalang (Satun province).

**Diagnosis.** A large species differing mainly from the other species in the structure of the male terminalia. Cercus brown, pointed, bearing a single apical bristle; cercus in lateral view slightly shorter than dorsal surstylus. Dorsal surstylus brown on apical border with short stout yellow bristles, ventrally with a dark spur-like apex (Fig. 10E). Fore tarsomere in male with an apical fork with outer prong a little longer than inner (anterior) prong.

**Distribution.** Satun province (Andaman Sea).

***Ngirhaphium meieri* Samoh & Grootaert, new species**  
(Figs. 7–9, 10D)

**Diagnosis.** A large species with apical segment of arista twice as long as basal aristal segment. Prongs on fore tarsomere 4 with exterior (posterior) prong sharply pointed longer than interior (anterior) prong. Apical tarsomere of fore leg with a black somewhat twisted prong below the posterior claw. Apical tarsomere of mid leg with a double dorsal comb of black squamiform bristles forming a flag. Mesonotum and abdominal tergites shining metallic green.

Cercus in lateral view slightly shorter than dorsal surstylus. Cercus brown, tip pointed bearing a single long, yellow bristle. Dorsal surstylus mushroom-shaped, yellowish brown, bordered with short, yellow bristles.

**Material examined.** HOLOTYPE ♂, labelled: THAILAND: Phang Nga province, Takuapa, Bang Yai, (8°54'27.5"N, 98°23'59.6"E), Samoh A, sweep netting, 9 February 2015

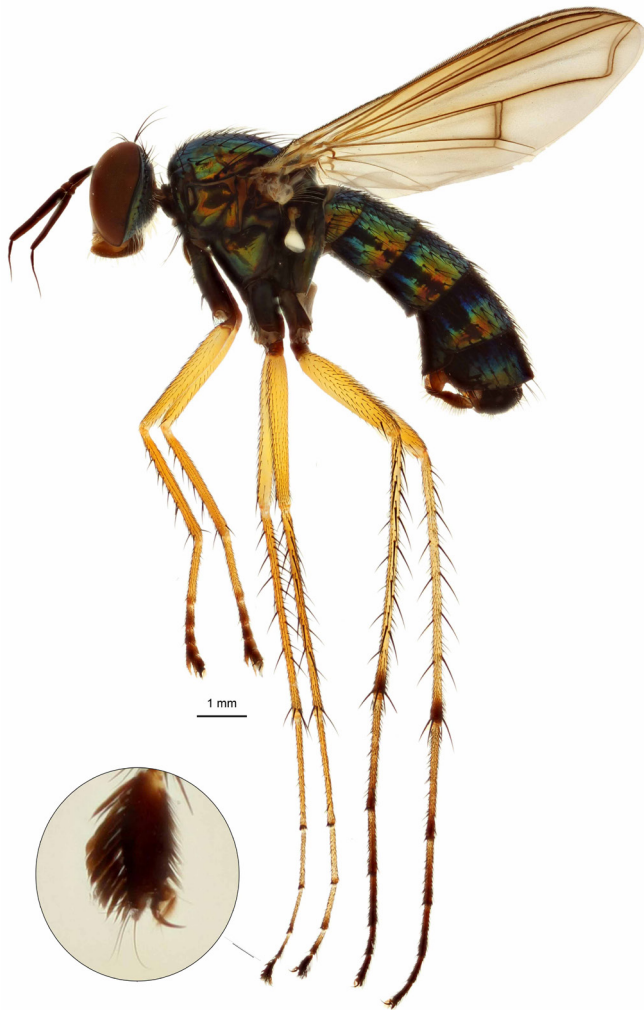


Fig. 6. *Ngirhaphium meieri*, new species, male habitus (photo: Abdulloh Samoh). Scale = 1 mm.

(PSUNHM). (Doli3 \_26\_005E, collected Abdulloh Samoh, PSUNHM).

**Paratypes.** 2 males; 2 females, Phang Nga province, Takuapa, Bang Yai, (8°54'27.5"N, 98°23'59.6"E), Samoh A, sweep netting, 9 February 2015 (PSUNHM); 3 females, Phang Nga province, Takuapa, Bang Yai, (8°54'27.5"N, 98°23'59.6"E), Samoh A, sweep netting, 13 July 2018 (PSUNHM); 1 male, Ranong province, Kraburi, Lam Liang, Phra Khayang cave (10°19'25.5"N 98°45'58.0"E), Samoh A., sweep netting, 10 July 2018 (PSUNHM).

**Etymology.** The species is dedicated to Rudolf Meier, head of Evolutionary Biology Laboratory, Department of Biological Sciences at the National University of Singapore (NUS), who provided us an opportunity to learn and do a molecular study of the marine dolichopodid flies.

**Description.** Male. (Fig. 6). Length body: 8.6–8.8 mm; wing: 8.4–8.6 mm. Head. Frons shining metallic green (not dusted), deeply excavated. Face greenish brown in ground-colour, silvery grey dusted; apex of face and clypeus yellowish brown in ground-colour, parallel-sided. Eyes pass beyond border of face; eyes densely set with minute white hairs.



Fig. 7. *Ngirhaphium meieri*, new species, female habitus. Scale = 1 mm.

Ocellar callus globular protruding from frons with two very long ocellars, directed backward, divergent. Vertical bristles long, half as long as ocellars, rather anterior on frons at level of ocellar callus, close to eye border, long, black, directed forward and cruciate. Pair of long black postverticals directed backward and crossing. Postoculars above strong, black in single row, below white and mixed with very long white hairs below mouth; postcranium greenish in ground-colour but grey dusted. Palpus long, strap-shaped, yellowish with few short black hairs, no apical bristle. Labella brown with black hairs. Antenna long, completely black. First segment long, 3 times as long as second segment; second segment short, apically with crown of short black bristles. Third segment very long strap-shaped, laterally flattened, with a lateral rim, about 6.5 times as long as width at base. Arista apical, apical segment twice as long as basal article, gradually tapering towards tip. Total length antenna: 2.1 mm. Length of scape: 0.39; pedicel: 0.13; postpedicel: 0.87; basal arista article: 0.23; apical arista article: 0.468 (all in mm).

Thorax and scutellum dark metallic green in ground-colour (bluish when seen from in front), covered with fine grey dusting. All hairs and bristles black. Pleura more densely grey dusted than mesonotum. Acrostichals biseriate, about 7 pairs, rows widening slightly behind. Presutural dorsocentrals multiseriate; 6 postsutural dorsocentrals: 4 short and 2 long prescutellars; 1 pair of strong scutellars. One long humeral with shorter bristle in front; 1 strong posthumeral, 2 strong notopleurals, 1 postsutural, 1 supraalar, 1 very strong postalar. Propleural bristles black, 6 short, fine upper and 2 lower propleural bristles (lower one twice as long as upper).



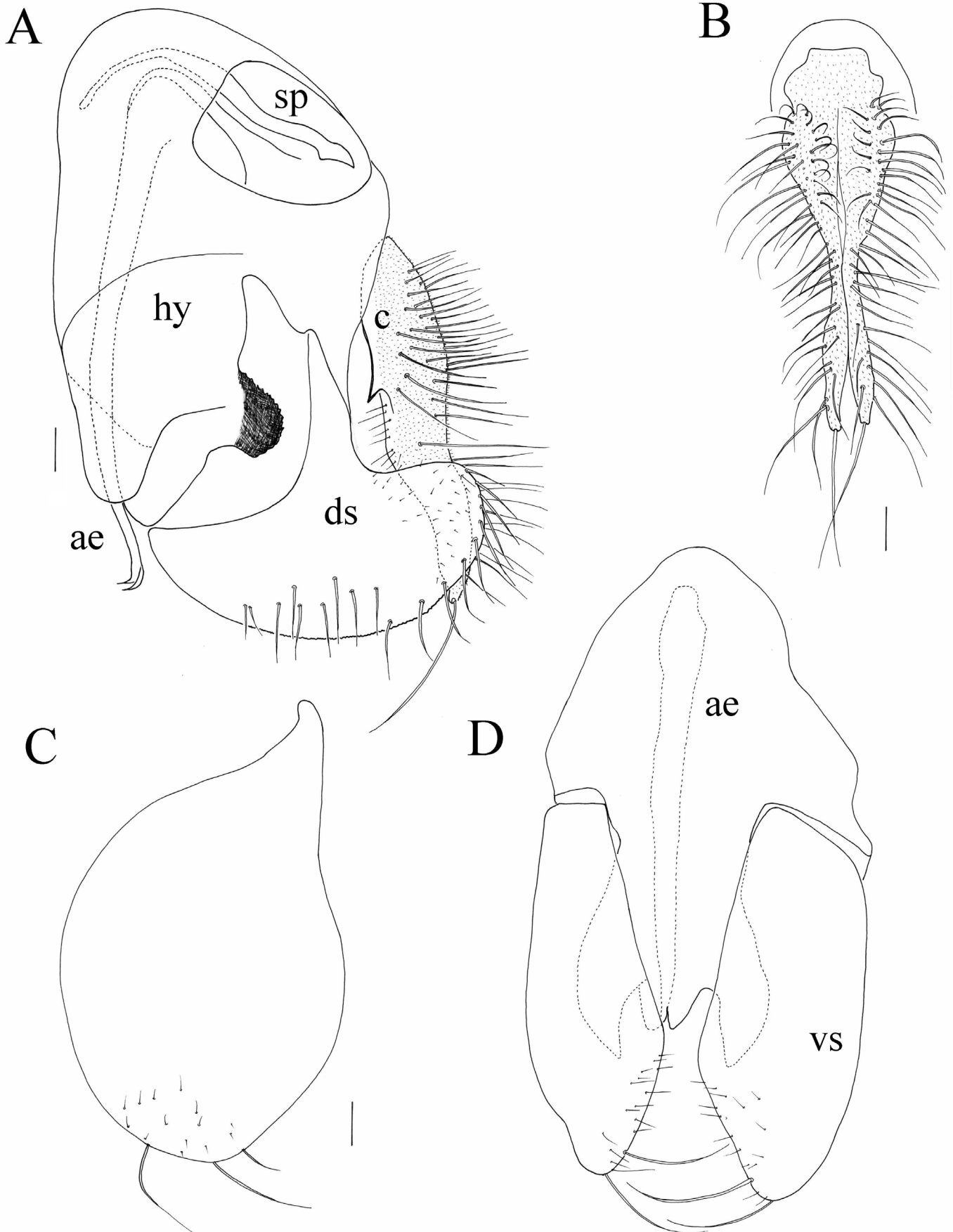


Fig. 8. *Ngirhaphium meieri*, new species, holotype male terminalia: A, Lateral view of genital capsule with left ventral surstylus removed; B, Cerci dorsally; C, Left ventral surstylus; D, Ventral view of genital capsule. Abbreviations: ae = aedeagus; c = cercus; ds = dorsal surstylus; hy = hypandrium; sp = sperm pump; vs = ventral surstylus. Scale = 0.1 mm.

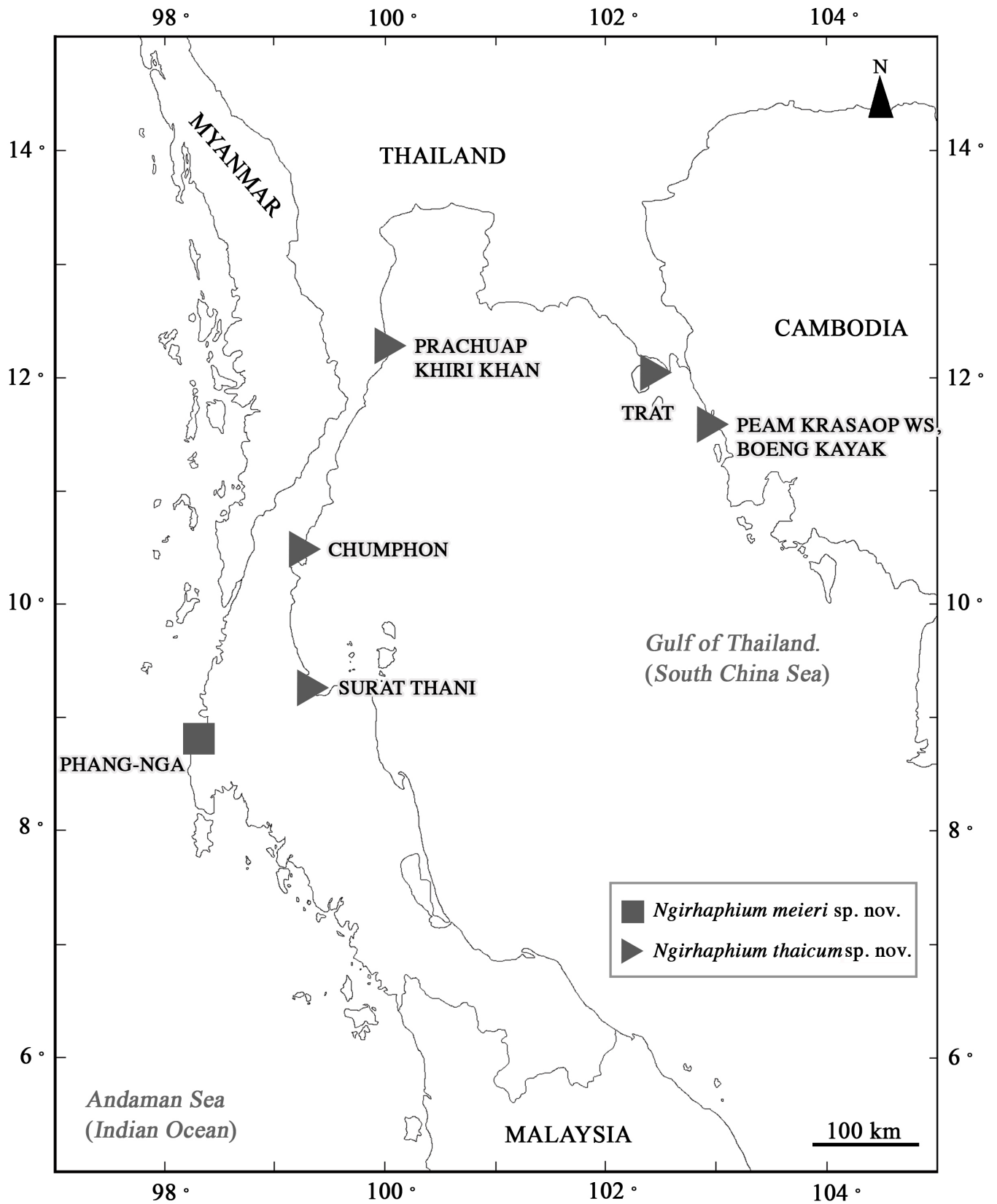


Fig. 9. Distribution map of *Ngirhaphium meieri*, new species and *Ngirhaphium thaicum*, new species.

Legs (Fig. 5) yellow, all bristles black. All coxae brownish black in ground-colour, covered with fine greyish dusting. All trochanters brown. Tip of hind tibia annulated brown at tip. Fore tarsus with tip of tarsomere 1 brownish, tarsomeres 2–4 almost entirely brown (especially dorsally), tarsomere 5 entirely black. Mid tarsus more yellowish than fore tarsus, with apical tarsomere black; hind tarsus black but base of tarsomere 1 and 2 paler.

Fore leg. Coxa with short black bristles. Fore femur slightly swollen on basal half; row of minute posteroventrals in apical half. Short preapical posterior bristle and 1 stronger anterior preapical bristle directed forward. Fore tibia with 4 strong ad, 4 strong pd and crown of 4 apicals. All tarsal segments densely set with black hairs and short black bristles. Tarsomere 4 with dorsal asymmetrical fork, extending over tarsomere 5; exterior (posterior) prong longer than interior (anterior) prong, both sharply pointed when seen in lateral view. Apical tarsomere with a black, blunt, somewhat twisted prong below the posterior claw, as long as apical tarsomere.

Two well-developed pulvilli and empodium present. Length of femur, tibia and tarsal segments (in mm): 1.6 : 1.62 : 0.8 : 0.26 : 0.2 : 0.16 : 0.2.

Mid leg. Coxa with short bristles anteriorly; no exterior bristle. Mid femur as wide as fore femur; ventrally with inconspicuous bristles; 1 strong anterior preapical and 2 tiny posterior preapicals. Tibia with 6 ad, 6 pd (might be considered as dorsal), 8 longer av and crown of long apicals. Tarsomeres 1–4 ventrally at tip with pair of short spine-like bristles as well as comb of shorter black spinules. Apical tarsomere of mid leg with a double, dorsal comb of black squamiform bristles forming a flag (Fig. 5 inset). Length of femur, tibia and tarsal segments (in mm): 1.9 : 2.4 : 1.3 : 0.74 : 0.54 : 0.3 : 0.3.

Hind leg. Coxa bare. Trochanter with a long dorsal bristle. Hind femur thicker than fore and mid femur, ventrally almost bare; 1 strong anterior preapical, 1 fine posterior preapical. Tibia stronger bristled than mid tibia with 7 long av, 7 ad, 7 pd and crown of long apicals. Tarsomeres 1–4 ventrally at tip with pair of short spine-like bristles as well as comb of shorter black spinules. Length of femur, tibia and tarsal segments (in mm): 1.8 : 3.3 : 1.4 : 0.8 : 0.64 : 0.36 : 0.4.

Wing (Fig. 6) tinged brownish (paler on imaged specimen), anteriorly between costa and  $R_{4+5}$  a little darker yellowish brown. Tp (crossvein r-m) brown seamed. Veins dark brown, yellowish at base.  $M_{1+2}$  sharply bent upwards and ending in costa closely near tip of  $R_{4+5}$ . Tp straight, a little longer than apical part of  $M_{3+4}$ . Anal vein reaching wing border. Haltere with white knob, stem a little infusate. Squama white, with long white cilia.

Abdomen subshining dark metallic green. Tergites densely set with quite long black bristles; hind-marginal bristles slightly longer than other bristles. Only tergites 1 and 5 with much longer marginal bristles. Sternites with very short hairs except for longer marginals on sternite 4.

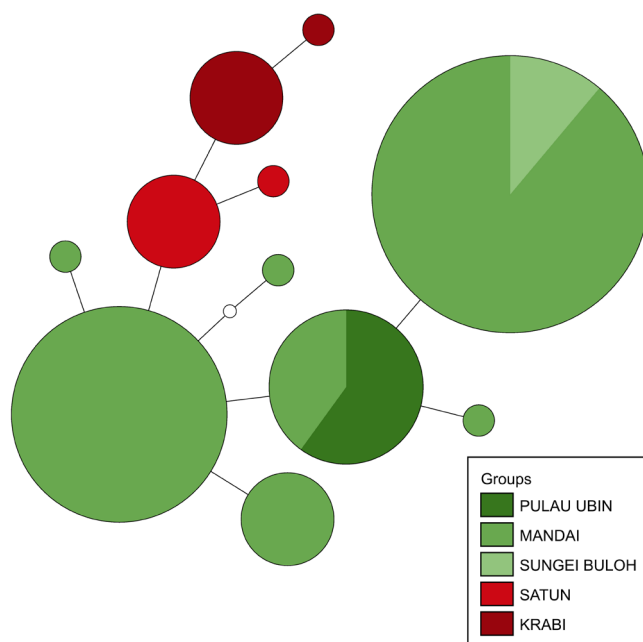


Fig. 10. Haplotype network of *Ngirhaphium murphyi*. Singapore: Pulau Ubin, Mandai Sungei Buloh; Thailand: Satun and Krabi.

Terminalia (Fig. 8). Cercus yellowish brown to brown, slightly shorter than dorsal surstylus (Fig. 8A). Both cerci look like fused for almost entire length, only tips free (Fig. 8B). Tip of cercus pointed, with a single yellow apical bristle, dorsally set with long blackish brown bristles, sometimes pale at tip. Dorsal surstylus brown, paler than cercus, with much widened tip in the shape of a mushroom, forming a transverse clasper on cercus, bordered with short but stout yellow bristles (Fig. 8A). Ventral and dorsal surstyli not fused. Ventral surstylus yellowish, oval (Fig. 8C), tip with 3 long bristles on apical border and a few short hair-like bristles at inside. Hypandrium dorsally split, with a large rounded black protuberance set with denticles (Fig. 8A).

Female. (Fig. 7). Body: 8.9–9.0 mm; Wing: 8.6–8.8 mm.

The female has larger labellae than the male, which is a characteristic of the genus.

The antenna is slightly shorter than in male with apical arisal segment twice as long as basal segment. Length of scape: 0.377; pedicel: 0.13; postpedicel: 0.741; basal arisal article: 0.234; apical arisal article: 0.455 (all in mm). Total length 1.94 mm.

Fore tarsomere 4 without dorsal fork. Tarsomere 5 with an apical dorsal projection and without ventral prong below the exterior claw. Mid tarsomere 5 without dorsal squamiform bristles.

**Distribution.** Phang Nga and Ranong provinces, Thailand, Andaman Sea (Indian Ocean).

**Remarks.** The new species is morphologically similar to *N. sivasothii* and even more to *N. chutamasa*. Differences can be found in the key to the Southeast Asian species here below.

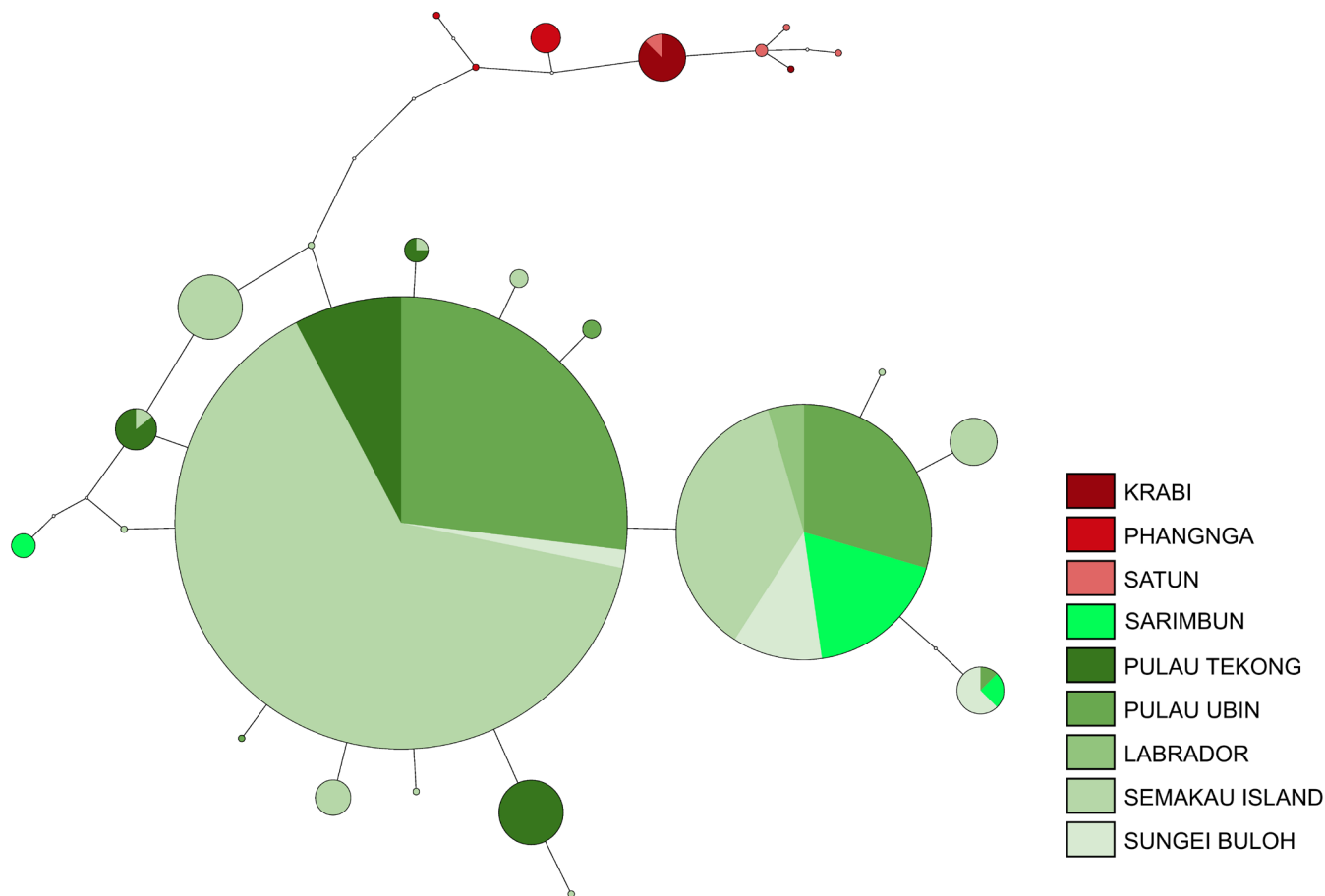


Fig. 11. Haplotype network of *Ngirhaphium sivasothii*. Thailand: Krabi, Phangnga, Satun; Singapore: Sarimbun, Pulau Tekong, Pulau Ubin, Labrador, Semakau Island, and Sungei Buloh.

***Ngirhaphium murphyi* Evenhuis & Grootaert, 2002**  
(Figs. 10, 12B)

*Ngirhaphium murphyi* Evenhuis & Grootaert, 2002: 310. Type locality: SINGAPORE: Kranji mangrove.  
*N. murphyi* Grootaert & Puniamoorthy, 2014: 147 (figs. 1–3, 4, 5, 18).

**Diagnosis.** A medium to large species (5.7–7.3 mm), generally with clear wings. Mesonotum and tergites metallic green. Apical arisal segment quite thick and short, only half as long as basal arisal segment. Male with cerci longer than surstyli and thus the tips are visible outside the surstyli.

**Material examined.** THAILAND: 1 ♂, 4 ♀, Satun province, Tarutao Island, Talo Wao bay (6°36'58.7"N 99°40'43.1"E), 11.viii.2014 (collected A. Samoh) (PSUNHM).

Krabi province: 1 male, 3 females, Ko Lanta, Ko Lanta Yai (7°38'40.9"N 99°02'42.3"E), Samoh A, sweep netting, 1 April 2018; Phang Nga province: 5 males, 1 female, Khuraburi, Phra Thong Island (9°03'04.8"N 98°15'56.8"E), Samoh A, sweep netting, 23 April 2018 (PSUNHM); 1 male, Takua Thung, Kalai, Ban Khuan Kalai (8°19'12.8"N 98°25'46.2"E); Samoh A, sweep netting, 14 July 2018 (PSUNHM); 1 male, Thappud, Ban Khuan Bosane pier (8°26'43.8"N 98°35'40.6"E); Samoh A, sweep netting, 15 July 2018 (PSUNHM).

Singapore: 37 specimens recorded from Mandai, Sungei Buloh and Pulau Ubin (35 barcoded; LKCNHM).

**Remarks.** The pedicel is yellowish-brown in females and black in males in southern Thailand. The pedicel is always black in both sexes in Singapore populations (Grootaert & Puniamoorthy, 2014).

**Distribution.** Singapore and South Thailand, Andaman Sea.

**Haplotype network.** Number of sequences used: 35  
Selected region: 1–313  
Number of sites: 313  
Number of haplotypes, h: 11.

***Ngirhaphium sivasothii* Grootaert & Puniamoorthy, 2014**  
(Figs. 11, 12A)

*Ngirhaphium sivasothii* Grootaert & Puniamoorthy, 2014: 150 (figs. 6–8, 9, 10, 17). Type locality: SINGAPORE: Semakau Island.

**Diagnosis.** A medium-sized species (4.5–5.5 mm), generally with dark infuscate wing and with longitudinal veins and Tp (posterior cross vein) brownish seamed. Mesonotum and tergites metallic green. Apical arisal segment shorter, but nearly as long as basal segment. Male with dorsal surstylus half as long as cerci, with a rectangular bend, set with very

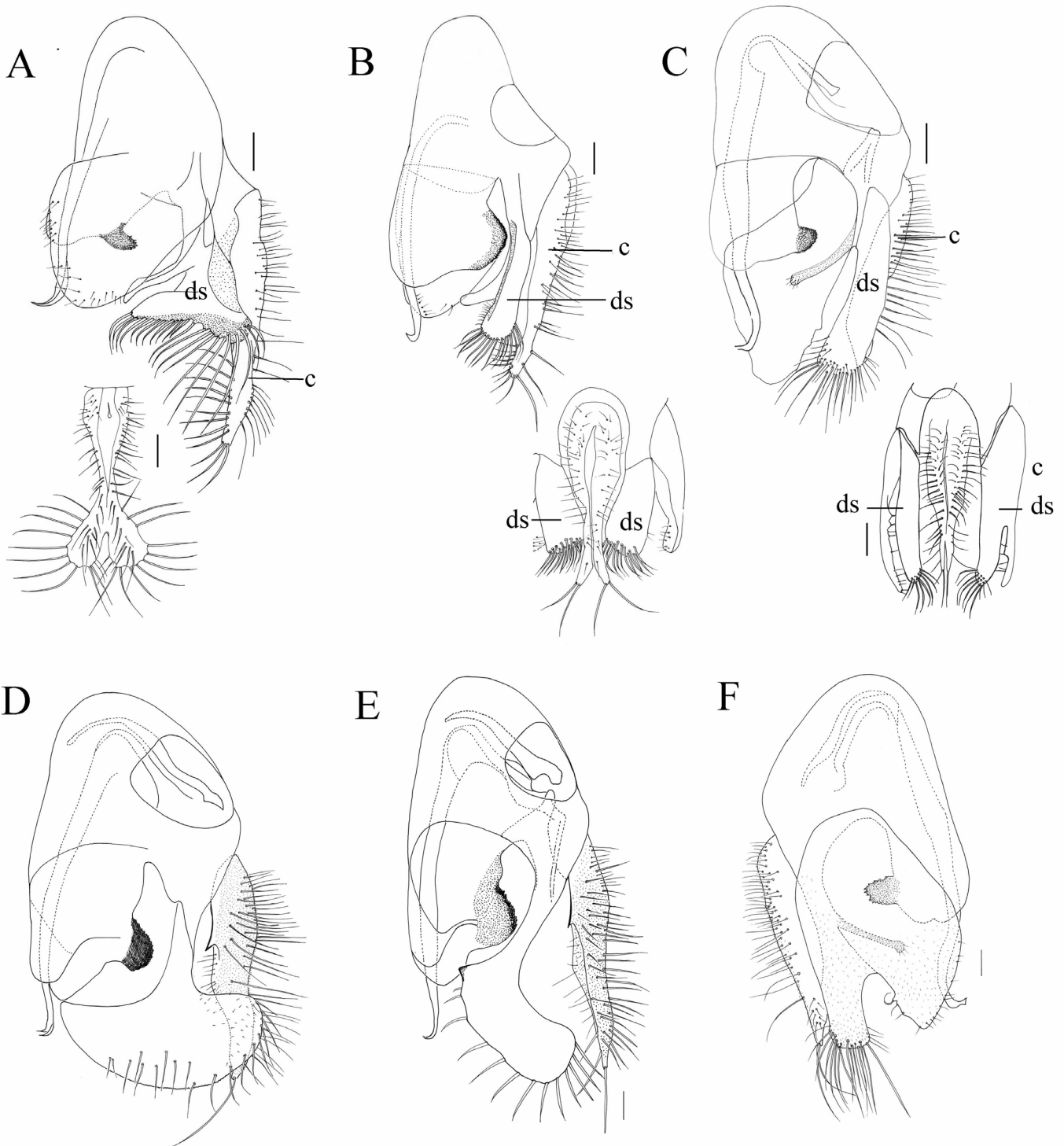


Fig. 12. Compilation of male terminalia of *Ngirhaphium* in lateral view. A, *Ngirhaphium sivasothii* left side with ventral surstylus removed; cerci dorsally; B, *Ngirhaphium murphyi* left side with ventral surstylus removed; cerci and dorsal surstyli dorsally; C, *Ngirhaphium caeruleum* left side with ventral surstylus removed; cerci and dorsal surstyli dorsally; D, *Ngirhaphium meieri*, new species left side with ventral surstylus removed; E, *Ngirhaphium chutamasae* left side with ventral surstylus removed; F, *Ngirhaphium thaicum*, new species right side. Scale = 0.1 mm.

long bristles (Fig. 12A). Cercus much longer than dorsal surstylus, tip wide, rounded, set with many long yellow bristles (Fig. 12A detail). Outer prong of apical fork on the fore tarsomere 4 slightly shorter than inner prong.

**Material examined.** THAILAND: 2 males, 1 female; Langu, Bakan Tothid (6°47'29.7"N 99°48'53.5"E), Samoh A, sweep netting, 1 June 2015, (PSUNHM); Krabi province: 3 males,

5 females; Muaeng, Sai Thai, Khlong Chilat (8°03'23.0"N 98°53'37.4"E); Samoh A, sweep netting, 21 February 2015 (PSUNHM); 1 male, 1 female; Koh Lanta, Khlong Yang (7°48'21.2"N 99°07'36.1"E); Samoh A, sweep netting, 13 June 2015 (PSUNHM); Trang province: 3 females; Kantang, Naklua, Ban Haad Saikhao (7°19'31.3"N 99°28'43.9"E); Samoh A, sweep netting, 15 July 2018 (PSUNHM); Phang Nga province: 2 males, 5 females; Thappud, Ban Khuan

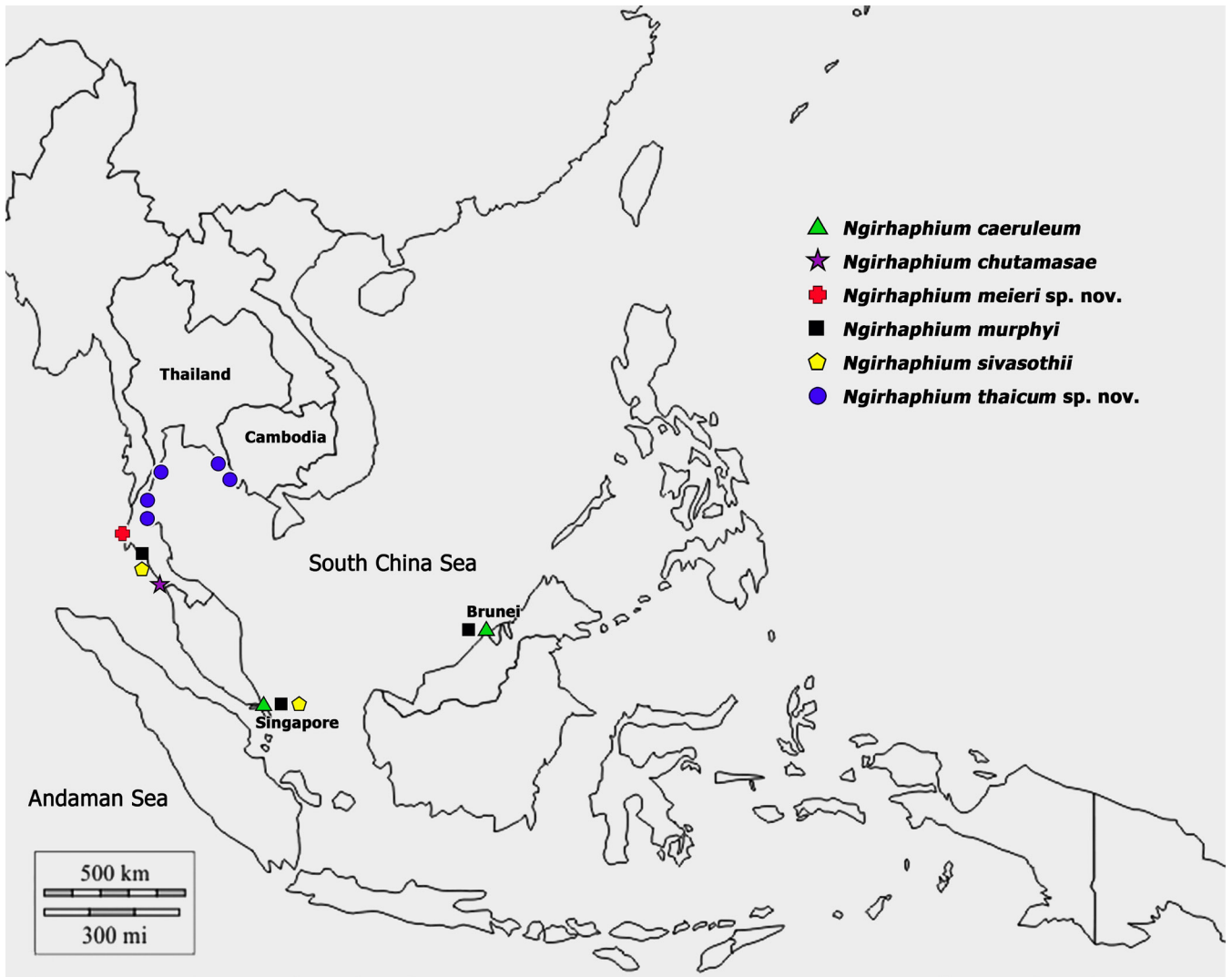


Fig. 13. Distribution map of *Ngirhaphium* in Southeast Asia

Bosane pier (8°26'43.8"N 98°35'40.6"E); Samoh A, sweep netting, 13 February 2015; 1 male, 2 females, Takuapa, Bangnaisi, Ban Bangdong (8°55'46.7"N 98°23'15.4"E), Samoh A, sweep netting, 11 February 2015 (PSUNHM); 14 females, Takuapa, Bangnaisi, Ban Bangnaisi (8°56'18.4"N 98°23'43.5"E), Samoh A, sweep netting, 11 February 2015 (PSUNHM); 1male, 14 females, Takuapa, Bangnaisi, Ban Bangnaisi (8°56'18.4"N 98°23'43.5"E), Samoh A, sweep netting, 12 February 2015 (PSUNHM); 1 male, Muang, Ban Bang Toei (8°21'54.4"N 98°34'50.9"E), Samoh A, Malaise trap, 13 February 2015 (PSUNHM); 2 males, 5 females, Takua Thung, Kalai, Ban Khuan Kalai (8°19'12.8"N 98°25'46.2"E); Samoh A, sweep netting, 14 July 2018 (PSUNHM); Ranong Province: 1 male, 4 females; Kraburi, Lam Liang, Phra Khayang cave (10°19'25.5"N 98°45'58.0"E), Samoh A., sweep netting, 10 July 2018 (PSUNHM).

Singapore: 3,127 specimens recorded (1,628 specimens NGS barcoded; LKCNHM).

**Distribution.** Singapore and South Thailand, Andaman Sea.

**Haplotype network.** Number of sequences used: 35  
Selected region: 1–313

Number of sites: 313  
Number of Haplotypes, h: 28

**Key to males of *Ngirhaphium* Evenhuis & Grootaert, 2002 from Southeast Asia**

1. Apical arisal segment half as long to almost as long as apical arisal segment.....2
- Apical arisal segment a little longer or more than 1.5 times longer than basal segment.....3
2. Apical arisal segment rather thick and half as long as basal segment; cerci pointed and longer than surstyli in lateral view (Fig. 12B); dorsal surstylus digitiform ..... *murphyi* Evenhuis & Grootaert
- Apical arisal segment broad at base and tapering toward tip, as long as basal arisal segment; cerci with apex much widened bearing numerous long bristles; apex dorsal surstylus very wide with numerous long bristles, surstylus much shorter than cerci (Fig. 12A) ..... *sivasothii* Grootaert & Puniamoorthy
3. Right dorsal surstylus fused with right ventral surstylus. Apical arisal segment 1.5 times as long as basal arisal segment: *caeruleum* complex .....4
- Right dorsal and ventral surstyli not fused .....5
4. Apex of ventral surstylus pointed (Fig. 2F) and the dorsal border bearing short tubercles; inside of ventral surstylus with

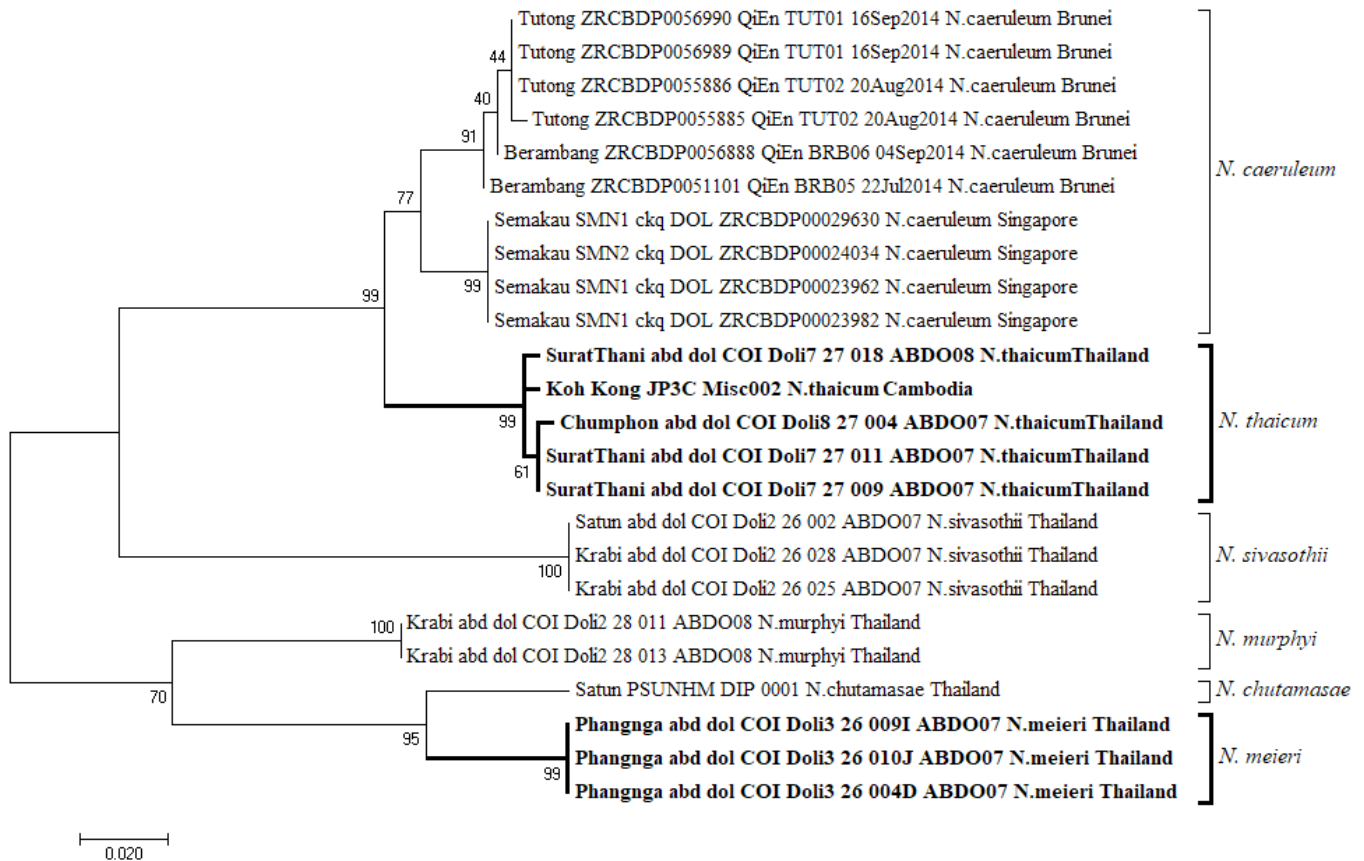


Fig. 14. Maximum likelihood tree of *Ngirhaphium* based on NGS barcodes (COI, 313bp).

- short pale bristles (Fig. 5B); thorax and abdomen with a green metallic shine (Thailand and Cambodia).....  
.....*thaicum*, new species
- Apex of ventral surstylus wider, not pointed (Fig. 2D) and the dorsal border smooth (Brunei) or if thorax and abdomen with a blue metallic shine some protuberances present (Singapore)..  
.....*caeruleum* Grootaert & Puniamoorthy
- 5. Dorsal surstylus with a wide tip, mushroom-shaped bearing a few rather thin, short bristles (Fig. 12B); apical aristal segment almost twice as long as basal segment.....*meieri*, new species
- Dorsal surstylus with a wide tip, rather sickle-shaped, bearing a rather thick yellow bristles on its apical border (Fig. 12E); apical aristal segment a little longer than basal segment .....  
.....*chutamasae* Samoh, Boonrotpong & Grootaert

## DISCUSSION

*Ngirhaphium* is a genus that occurs in the mudflats of the front mangrove and also follows the creeks inside the back mangrove. They are often observed hovering over the mudflats and displaying in sunny patches.

There are currently six described species of *Ngirhaphium*, all confined to Southeast Asia. The upper northern limit of the distribution of *Ngirhaphium* in the South China Sea is probably the southern coasts of Cambodia and Vietnam. Evidence for this is that *Ngirhaphium* was not recorded during a recent extensive survey of the mangroves of Hong Kong (Grootaert, unpublished) and was neither recorded in the Philippines (Ramos et al., 2018). No information of its southern east limits is yet available since the mangroves

of Indonesia and New Guinea have not been explored intensively.

Remarkable is that the coast of the Andaman Sea in peninsular Thailand is actually the most diverse with four species: *N. chutamasae*, *N. meieri*, new species, *N. murphyi* and *N. sivasothii*. It is likely that more species will be found on the northern coasts of the Andaman Sea e.g. in Myanmar with the numerous islands and pristine mangroves as suggested by the high diversity on the Andaman Sea coast of Thailand. The high diversity along the Andaman Sea coast of species occurring in the front mangrove is observed also in the genus *Thinophilus*, where 17 species are recorded and of these, 13 are only known from this region up to now. Eleven species are found on the coasts of the Gulf of Thailand, seven being exclusive for this region (Samoh et al., 2019).

Singapore that lays on the meeting point of the Andaman Sea and the South China Sea has three species, *N. caeruleum*, *N. murphyi*, and *N. sivasothii*. *Ngirhaphium caeruleum* seems to form a species complex composed of *N. caeruleum* and *N. thaicum*, new species. This species complex occurs only in the South China Sea and is hitherto the only species known from the South China Sea. There are numerous observations on the East coast of Singapore of the metallic blue species but on the smaller islands at the East of Singapore (Pulau Ubin and Pulau Tekong) a metallic green form occurs that is very similar to the specimens observed in Brunei as can be seen in the haplotype network of the *caeruleum* complex (Fig. 1). Remarkable is that the small differences in the NGS barcode seem to be reflected in differences in the structure

of the surstyli or male clasping organ. *Ngirhaphium murphyi* is present mainly on the West coast of Singapore and very rare on the East coast suggesting that the population is mainly present on the coasts of the Andaman Sea and did not disperse well around the island of Singapore towards the South China Sea.

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**APPENDIX**

Selected NGS barcodes of the six *Ngirhaphium* species in Southeast Asia.

Below are the main aligned haplotypes of the species. They can be copied/pasted in a fasta file so that the position of newly sequenced material will appear immediately.

>Phangnga\_abd\_dol\_COI\_Doli3\_26\_009I\_ABDO07\_N. meieri\_Thailand  
CCTATCCGCAGGAATTGCCACGGAGGGGCATCTGTAGACCTAGCTATTTTCTCCCTTCACTTAGCAGGTATTT  
CATCAATTCTAGGAGCAGTTAATTTTATTACAACAGTAATCAATATACGATCCACAGGAATTACATTTGATC  
GAATACCCCTATTTGTTTGATCCGTAGTAATTACTGCAATTCTACTCCTCCTTTCCCTTCCCGTACTTGCAGGA  
GCAATCACAATACTTCTAACAGACCGAAACTTAAACACATCATTTTTTCGACCCAGCAGGAGGAGGTGACCCAA  
TTCTCTACCAACACCTATTC

>Phangnga\_abd\_dol\_COI\_Doli3\_26\_010J\_ABDO07\_N. meieri\_Thailand  
CCTATCCGCAGGAATTGCCACGGAGGGGCATCTGTAGACCTAGCTATTTTCTCCCTTCACTTAGCAGGTATT  
TCATCAATTCTAGGAGCAGTTAATTTTATTACAACAGTAATCAATATACGATCCACAGGAATTACATTTGATC  
GAATACCCCTATTTGTTTGATCCGTAGTAATTACTGCAATTCTACTCCTCCTTTCCCTTCCCGTACTTGCAGGAG  
CAATCACAATACTTCTAACAGACCGAAACTTAAACACATCATTTTTTCGACCCAGCAGGAGGAGGTGACCCAATT  
CTCTACCAACACCTATTC

>Phangnga\_abd\_dol\_COI\_Doli3\_26\_004D\_ABDO07\_N. meieri\_Thailand  
CCTATCCGCAGGAATTGCCACGGAGGGGCATCTGTAGACCTAGCTATTTTCTCCCTTCACTTAGCAGGTATTT  
CATCAATTCTAGGAGCAGTTAATTTTATTACAACAGTAATCAATATACGATCCACAGGAATTACATTTGATCG  
AATACCCCTATTTGTTTGATCCGTAGTAATTACTGCAATTCTACTCCTCCTTTCCCTTCCCGTACTTGCAGGAGCA  
ATCACAATACTTCTAACAGACCGAAACTTAAACACATCATTTTTTCGACCCAGCAGGAGGAGGTGACCCAATTCTC  
TACCAACACCTATTC

>Satun\_abd\_dol\_COI\_Doli2\_26\_002\_ABDO07\_N. sivasothii\_Thailand  
TCTATCTGCAGGAATTGCCATGGAGGAGCCTCAGTAGACCTAGCAATCTTCTCTTTGCATTTAGCAGGTATTT  
CATCAATTCTAGGAGCAGTAAATTTTATTACAACAGTAATTAATATACGATCCACAGGAATTACCTTTGATC  
GAATACCCCTATTTGTATGATCAGTAGTAATTACTGCAATCCTACTTCTTCTTTCTCTCCCAGTTTTAGCCGGAG  
CTATTACAATACTCCTAACAGACCGAAACTTAAATACCTCATTTTTTTGACCCAGCAGGAGGAGGAGATCCAATCC  
TTTACCAACATCTATTC

>Krabi\_abd\_dol\_COI\_Doli2\_26\_028\_ABDO07\_N. sivasothii\_Thailand  
TCTATCTGCAGGAATTGCCATGGAGGAGCCTCAGTAGACCTAGCAATCTTCTCTTTGCATTTAGCAGGTATTT  
CATCAATTCTAGGAGCAGTAAATTTTATTACAACAGTAATTAATATACGATCCACAGGAATTACCTTTGATC  
GAATACCCCTATTTGTATGATCAGTAGTAATTACTGCAATCCTACTTCTTCTTTCTCTCCCAGTTTTAGCCGGAG  
GCTATTACAATACTCCTAACAGACCGAAACTTAAATACCTCATTTTTTTGACCCAGCAGGAGGAGGAGATCCAATC  
CTTTACCAACATCTATTC

>Krabi\_abd\_dol\_COI\_Doli2\_26\_025\_ABDO07\_N. sivasothii\_Thailand  
TCTATCTGCAGGAATTGCCATGGAGGAGCCTCAGTAGACCTAGCAATCTTCTCTTTGCATTTAGCAGGTATTT  
CATCAATTCTAGGAGCAGTAAATTTTATTACAACAGTAATTAATATACGATCCACAGGAATTACCTTTGATC  
GAATACCCCTATTTGTATGATCAGTAGTAATTACTGCAATCCTACTTCTTCTTTCTCTCCCAGTTTTAGCCGGAG  
GCTATTACAATACTCCTAACAGACCGAAACTTAAATACCTCATTTTTTTGACCCAGCAGGAGGAGGAGATCCAATC  
CTTTACCAACATCTATTC

>Krabi\_abd\_dol\_COI\_Doli2\_28\_011\_ABDO08\_N. murphyi\_Thailand  
CCTATCTGCAGGAATTGCCACGGAGGAGCCTCAGTAGACTTAGCTATTTTTTCCCTTCACTTAGCAGGTATCT  
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