Postdoc Fellowships for non-EU researchers

Final Report

Name	Piyatida PIMVICHAI
Selection	2012
Host institution	RBINS – Royal Belgian Institute of Natural Sciences
Supervisor	Thierry Backeljau
Period covered by this report	Fellowship:15/02/2014 to 31/01/2015 + 01/06 to 15/11/2015 RBINS extension:16/11/2015 to 15/02/2016
Title	Taxonomy and Phylogeny of the Millipede Family Pachybolidae with Emphasis on the Southeast Asiatic Fauna

1. Objectives of the Fellowship

The millipede (Diplopoda) family Pachybolidae occurs in tropical Asia, Africa, Madagascar, Australia, and South America. It comprises some 50 genera and a couple of hundred species, most of which were only very recently described from Africa and Madagascar. In contrast, Asian pachybolids have received little attention. The related family Pseudospirobolellidae is mainly distributed in SE Asia, Africa, Madagascar and the Society Islands. Yet, only two genera and four species (with a large number of synonyms) of Pseudospirobolellidae are currently recognized as valid. Given the very local distribution of most millipede species, and based on unpublished field observations in SE Asia, and particular Thailand, it has become clear that a large number of pachybolid and pseudospirobolellid species remain to be characterized and need to be formally described. As such, both families are in need of taxonomic revision.

Against this background, the primary objectives of this fellowship were: (1) to revise the Thai fauna of Pachybolidae and Pseudospirobolellidae, taking into account the fauna of neighbouring countries, (2) to produce and apply DNA barcodes to explore species limits and hence provide a DNA identification tool, and (3) to estimate phylogenetic relationships within and among the Pachybolidae and Pseudospirobolellidae on a worldwide scale in order to understand morphological character evolution and biogeographic patterns.

In addition to these primary objectives, the fellowship was also used to revise and disentangle a series of cryptic species complexes in a third family, viz. the Harpagophoridae. This was done by the same integrative approach as used for the Pachybolidae and Pseudospirobolellidae.

2. Methodology in a nutshell

Without going into detail, following methods were applied in the course of this fellowship:

[1] Fieldwork in Thailand to collect fresh specimens of Pachybolidae and Pseudospirobollelidae (20/08-07/09/2014; see [3] under heading 5).

[2] Study of historical taxonomic collections (including type specimens) in natural history museums (RBINS, RMCA, NHM Vienna, NHM Copenhagen, NHM London, MNHN Paris, MNHN Geneva, Chulalongkorn University) either by visiting the museums concerned (see also [5] and [6] under heading 5) or by borrowing the required material for study at RBINS.

[3] Extensive morphological characterization of the genital anatomy (male and female) of all specimens surveyed in museum collections and of the freshly collected material that was used for DNA analysis. This morphological work relied on observations using a stereomicroscope and stacking microphotography, as well as Environmental Scanning Electron Microscopy. Detailed drawings of the genital parts were produced to complement the photographic documentation.

[4] Classical Sanger DNA sequencing analysis of mitochondrial COI and 16S rDNA, as well as nuclear 18S rDNA of Pachybolidae, Pseudospirobolellidae, and Harpagophoridae, involving DNA extraction, PCR amplification using universal COI, 16S rDNA and 18S rDNA primers, PCR product purification, and DNA Sanger sequencing using the ABI 3130xl automated capillary DNA sequencer at RBINS. Sequences were aligned and used for taxonomic (DNA barcoding) and phylogenetic (Maximum Likelihood and Bayesian Inference) analysis by means of various software.

[5] Restriction Site Associated DNA sequencing (RAD-seq) of 16 specimens of six species of the genus *Thyropygus* and two species of the genus *Anurostreptus* in the family Harpagophoridae. Preparation of DNA libraries and subsequent treatment of the sequence data. RAD-seq sequences were produced at the VIB facility of the University of Antwerp. This work is still in progress.

[6] Mitogenomics (complete mitochondrial DNA genomes) of cryptic harpagophorid species, particularly in the genus *Thyropygus*. The mitogenome of millipedes is estimated to have a length of about 15,000 bp and thus represents a considerable number of nucleotide positions. This, together with the gene order in the mitogenome, represents a wealth of phylogenetic and taxonomic information. Currently, Dr. Pimvichai is establishing the mitogenomes of eight harpagophorid species. The preparation of the DNA samples for complete mtDNA sequencing has been done by Dr. Pimvichai, the mitogenome sequencing has been outsourced to BGI (Beijing Genomics Institute). This work is still in progress.

[7] Submission of all DNA sequences to the GenBank and BOLD databases. All nomenclatural acts are registered at ZooBank. Type material of newly described species and all voucher specimens analyzed (morphology and/or DNA sequencing) are deposited in both Thai (e.g. Museum of Zoology of Chulalongkorn University, Bangkok) and European (e.g. Natural History Museum of Denmark, Copenhagen; RBINS) natural history collections.

3. Results

We have been focusing on the morphological differentiation and DNA sequence analysis of populations (and eventually species) within the families Pachybolidae, Pseudospirobolellidae and Harpagophoridae. Based on the morphology of the gonopods (male sexual organs) and vulvae (female sexual organs) and DNA sequence data we discovered more than 10 new species of Pachybolidae, seven new species of Pseudospirobolellidae, and nine new species of Harpagophoridae, from Thailand, Laos, Viet-Nam and Malaysia. As such the number of pachybolid and pseudospirobolellid species in the study area has increased by more than 13% ! Some examples of new pachybolid and pseudospirobolellid species are shown in Figs 1-4.

Fig. 1. External morphology of live pachybolid and pseudospirobolellid millipedes

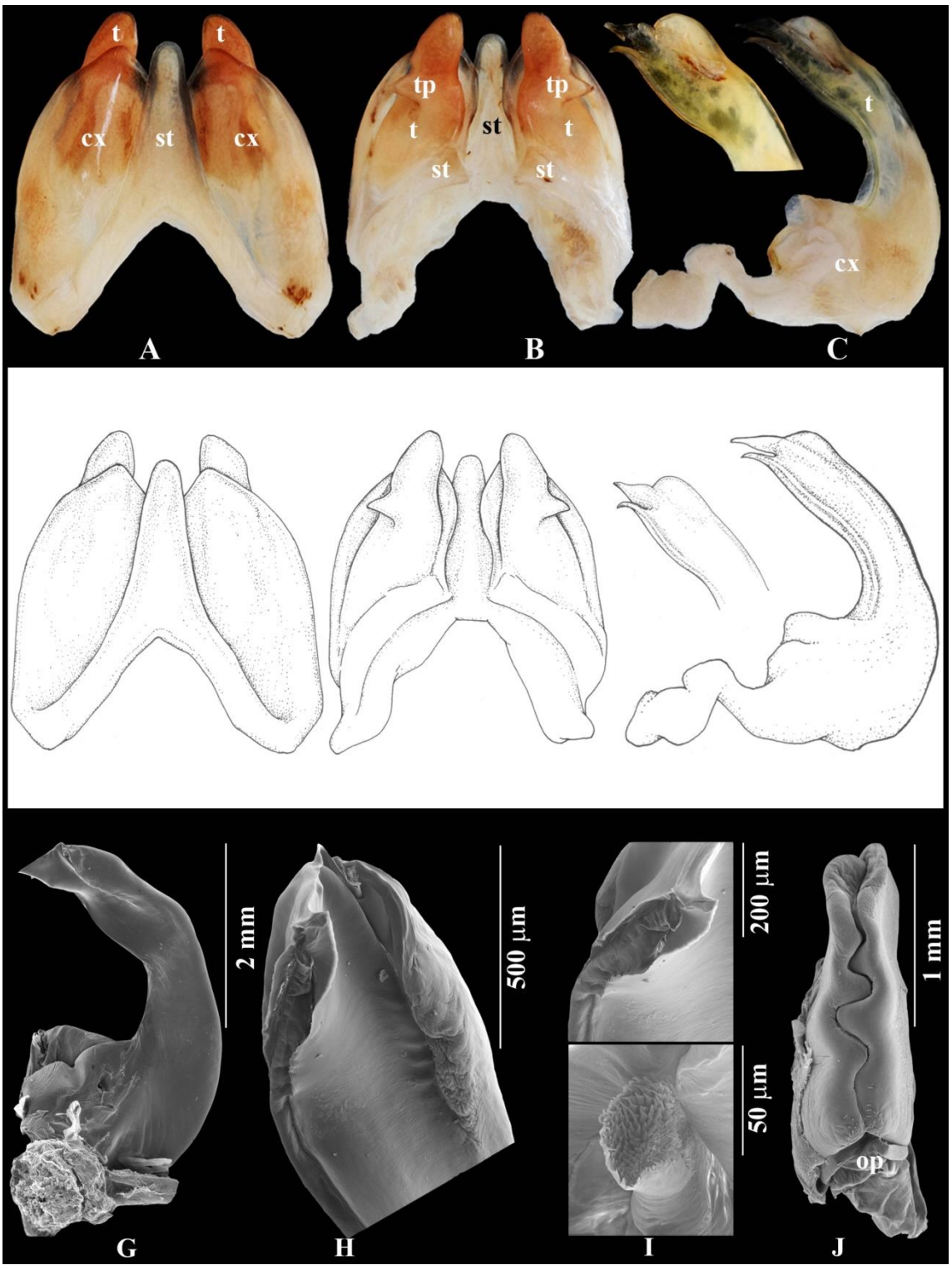

Fig. 2. Male gonopods (A-I) and female vulvae (J) of *Tonkinbolus* n. sp. 6., from Sriwilai Temple (SVL). See Fig. 1 for the living animal.

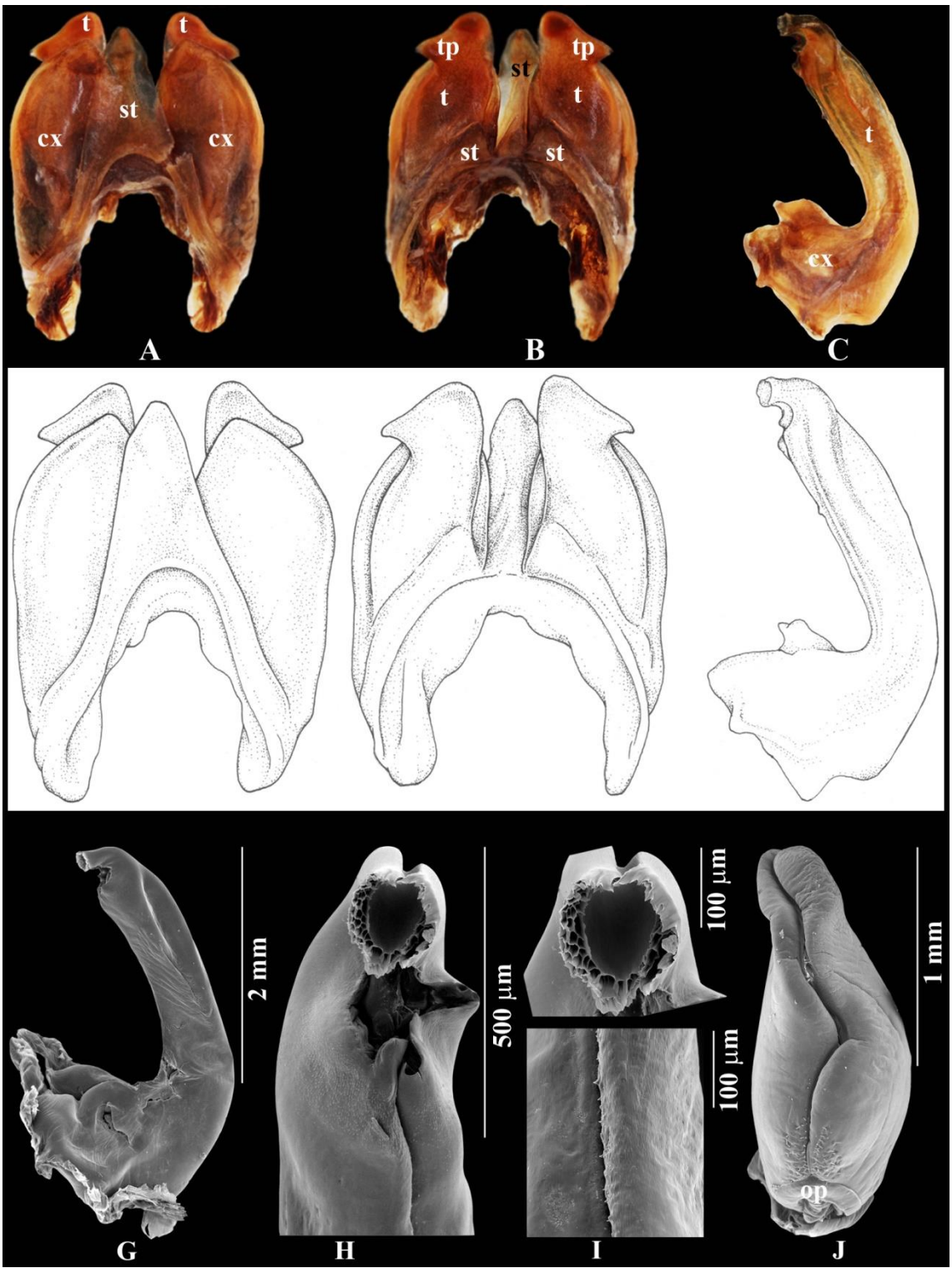

Fig. 3. Male gonopods (A-I) and female vulvae (J) of *Tonkinbolus* n. sp. 1, from Khao Chong-Bantad (CPP). See Fig. 1 for the living animal.

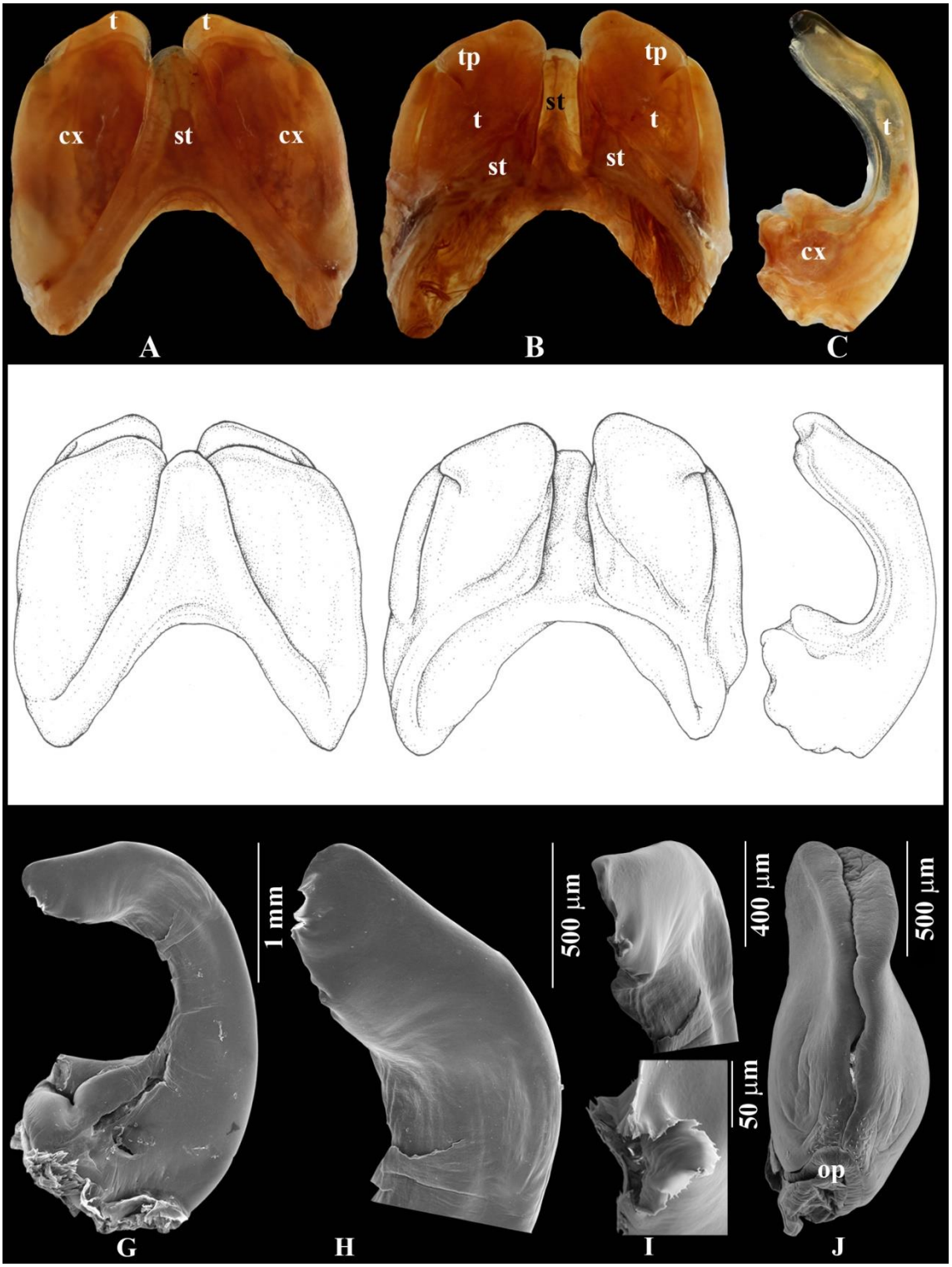

Fig. 4. Male gonopods (1-I) and female vulvae (J) of *Tonkinbolus* n. sp. 2., from Koh Sam-Sao (KSS). See Fig. 1 for the living animal.

Thai pachybolid specimens mostly belong to the genus *Tonkinbolus*. However, after the type species of *Tonkinbolus* viz., *T. scaber*, was examined we found that it is similar to *Litostrophus segregatus*, which is the single species in the monotypic genus *Litostrophus*. These two species are extremely similar in gonopod and vulvae morphology. The other seven *Tonkinbolus* species differ from the type species by having a laterad triangular process protruding from the lateral margin at ca. 2/3 of its height on the anterior gonopods in posterior view. Obviously, the identity of the type species of *Tonkinbolus* needed further scrutiny, using DNA sequence data.

The pachybolid and pseudospirobolellid DNA sequence data involved COI sequences of 91 specimens, representing 22 nominal species and 36 unknown specimens (supposed to be new species). *Narceus americanus* and *Floridobolus penneri* were used as outgroup taxa. The 16S rDNA data involved 80 specimens, representing 21 nominal species and 27 unknown specimens. Pseudospirobolellid species were used as outgroup taxa. The concatenated data (COI+16S rDNA) involved 67 specimens, representing 16 nominal species and 24 unknown specimens.

The COI (Fig. 6), 16S rDNA (Fig. 7) and concatenated COI+16S rRNA (Fig. 5) trees were topologically identical (compare Figs 5-7). They strongly supported the monophyly of the families Pachybolidae and Pseudospirobolellidae. We use the tree of the concatenated sequences (Fig. 5) to pinpoint a number of general observations, while the COI tree (Fig. 6) will be used to illustrate the power of DNA barcoding to distinguish cryptic taxa.

The Pseudospirobolellidae clade was divided into three subclades, viz. *Benoitolus* (about five new species), two species of *Pseudospirobolellus* and an unknown Pseudospirobolellid taxon (TPT1) (Fig. 5). Conversely, the Pachybolidae clade underpinned the monophyly of several morphology based genera, such as *Tonkinbolus*, *Litostrophus* and *Pelmatojulus*. The upper clade includes species of the subfamily Trigoniulinae (which was formerly considered as a separate family, but our data clearly show that Trigoniulinae is part of the Pachybolidae), whereas the lower clade coincides with the subfamily Pachybolinae from Africa, Madagascar, India and SE Asia (Fig. 5). The clade comprising the genera *Tonkinbolus* and *Litostrophus* was maximally supported, while the monophyly of either genus was also strongly supported.

Yet, the monophyly of the genus *Tonkinbolus* was not 'complete' since the *Tonkinbolus* clade also comprised *Aulacobolus rubropunctatus*, rather than that this latter species was joined in a clade with *Aulacobolus uncopygus*, the sister taxon of the *Tonkinbolus* + *Litostrophus* clade (Figs 5-6). In fact, *Aulacobolus rubropunctatus* even made *Tonkinbolus dollfusii* a paraphyletic species ! Conversely, the 16S rDNA tree (Fig. 7) showed that *Tonkinbolus scaber*, the type species of *Tonkinbolus*, appears as sister taxon to *Litostrophus*, outside the *Tonkinbolus* clade!

It is beyond the scope of this report to discuss all taxonomic and evolutionary aspects of these trees. Yet, just by way of example we illustrate here the ability of DNA barcoding based on COI sequences to separate cryptic taxa. Indeed, in Figs 1-4 we show the external morphology and genital parts of three cryptic *Tonkinbolus* species, provisionally labelled as CPP (species 1), KSS (species 2) and SVL (species 6). From Fig. 1 it will be clear that with respect to their overall external morphology, these three taxa are indistinguishable or nearly so. Yet, their genital features do suggest a number of seemingly diagnostic characters, even if at this level the interpretation remains difficult (Figs 2-4). But, when looking at the positions of the three taxa in the COI tree (Fig. 6), then the tree taxa are widely separated in three distant, well-supported clades. This result strongly supports their interpretation as three different (phylogenetic) species.

With respect to the taxonomic work on the Harpagophoridae, we refer to the publication in Attachment 2.

Finally, we attempted to establish 18S rDNA sequences for all specimens, though this was only successful in a minority of the species. Therefore we do not deal with these data here. Yet, it is planned for the future collaboration to explore this issue further.

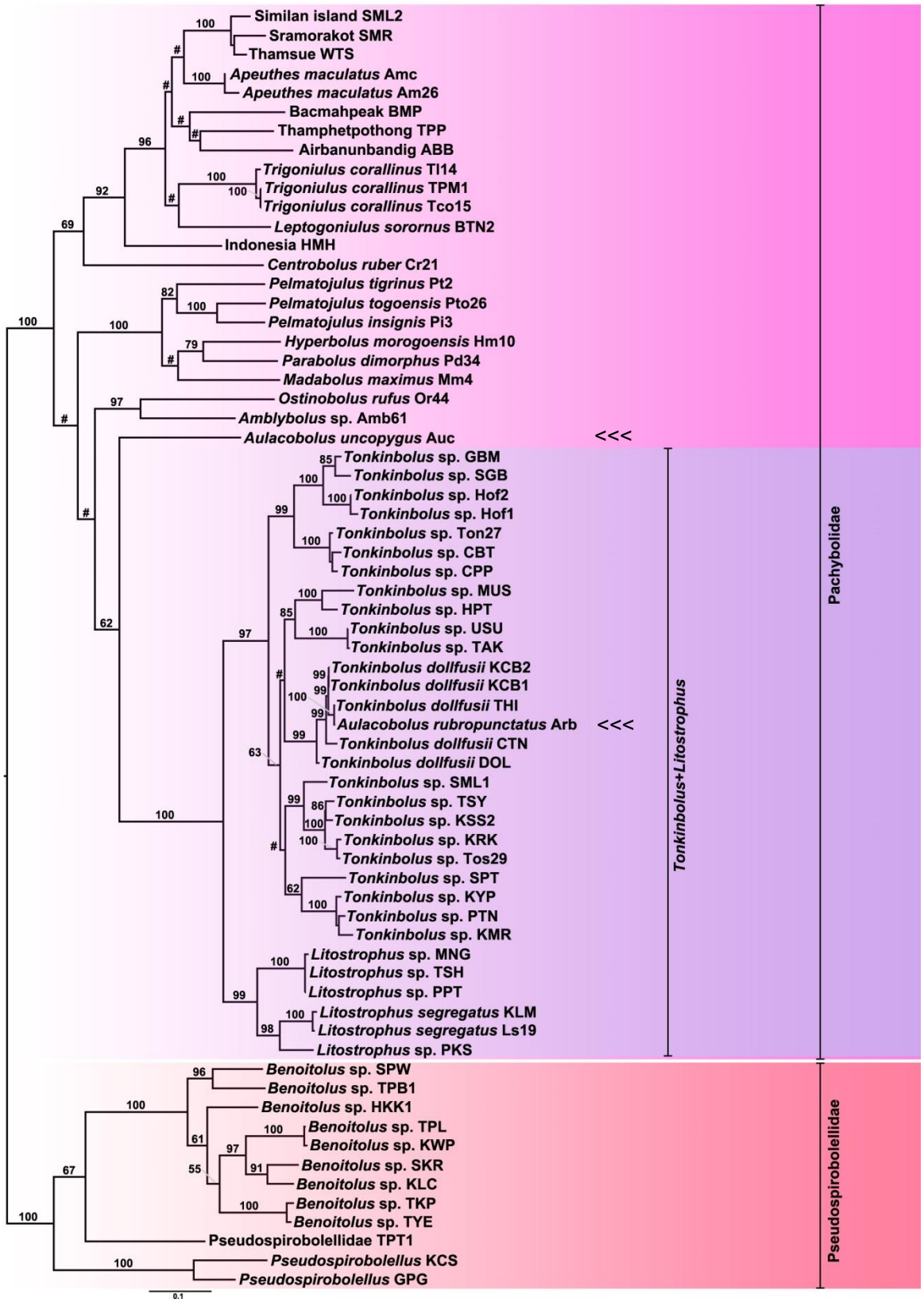

Fig. 5. Phylogenetic relationships of Pachybolidae and Pseudospirobolellidae species based on maximum likelihood analysis of concatenated COI (660 bp) and 16S rDNA (533 bp) sequences. Numbers at nodes indicate bootstrap support values. # indicates branches which received < 50% bootstrap support.

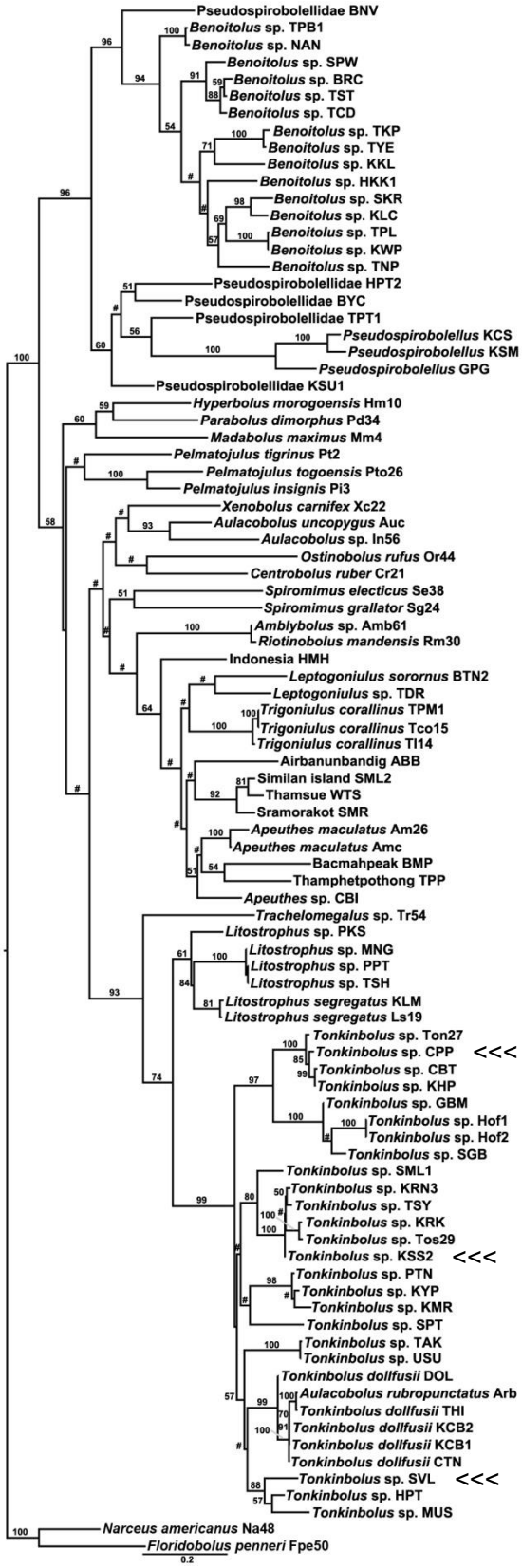

Fig. 6. Phylogenetic relationships of Pachybolidae and Pseudospirobolellidae species based on maximum likelihood analysis of COI (660 bp) sequences. Numbers at nodes indicate bootstrap support values. # indicates branches which received < 50% bootstrap support. Note the positions of CPP, KSS and SVL.

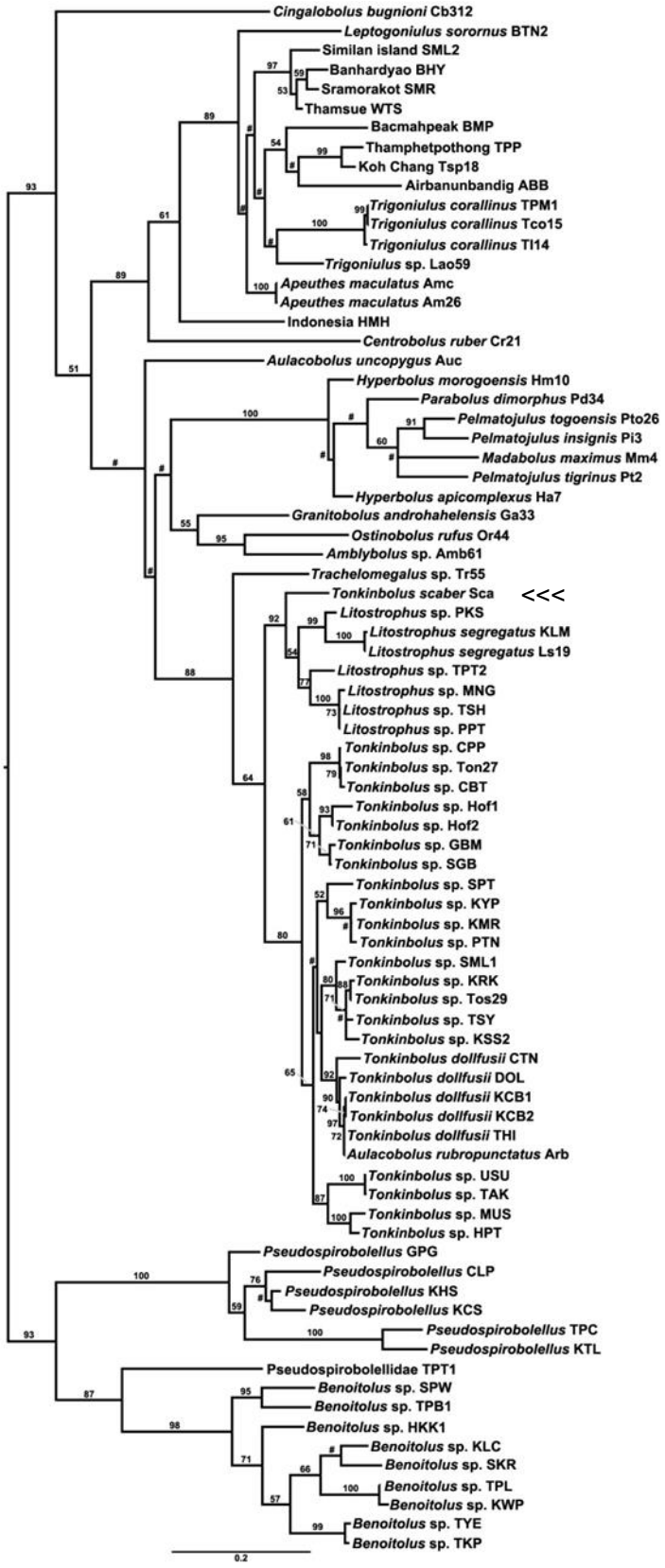

Fig. 7. Phylogenetic relationships of Pachybolidae and Pseudospirobolellidae species based on maximum likelihood analysis of 16S rDNA (533 bp) sequences. Numbers at nodes indicate bootstrap support values. # indicates branches which received < 50% bootstrap support. Note the position of *Tonkinbolus scaber* as sister taxon to *Litostrophus*, rather than as member of the *Tonkinbolus* clade.

Some taxonomic implications:

According to the DNA sequence data *Litostrophus* and *Tonkinbolus* were separated into two different clades. *Tonkinbolus scaber*, the type species of *Tonkinbolus* came out together with *Litostrophus* (see 16S rDNA tree in Fig. 7), which is congruent with gonopodal characters. Hence *Litostrophus* and *Tonkinbolus* are synonymous and because the name *Tonkinbolus* is older than *Litostrophus*, it has priority over the latter, so that *Litostrophus segregatus* should now be referred to as *Tonkinbolus segregatus*.

The synonymization of *Tonkinbolus* and *Litostrophus* also means that a new genus name is needed for the remaining '*Tonkinbolus*' clade. *Atopochetus* is a valid, alternative name that might be used to this end. *Atopochetus rubrodorsalis* was described by Attems in 1953 as the type species of *Atopochetus*. Later in 1962, Hoffman treated *Atopochetus rubrodorsalis* as a junior synonym of *Aulacobolus rubropunctatus* Attems, 1938. Our morphological and DNA sequence data from the type specimens and other material showed that *Aulacobolus rubropunctatus* and *Tonkinbolus dollfusii* (Pocock, 1893) should treated as a single species, with the latter species name having priority over the former. As such it seems indeed warranted to apply the name *Atopochetus* to the remaining *Tonkinbolus* clades.

Another *Aulacobolus* species that has been reported from SE Asia is *Aulacobolus brevipygus* Golovatch & Korsós, 1990. This species is, like *Aulacobolus rubropunctatus*, definitely different from all other (10) *Aulacobolus* species, which occur in India. Therefore, the genus *Aulacobolus* (type species *Aulacobolus uroceros*) seems to be endemic to the Indian fauna and does not occur in SE Asia.

Obviously the preceding nomenclatural and taxonomic issues need to be definitively solved and published. This is planned as part of the future collaboration between the Thai and Belgian partners. In addition, our phylogenetic trees show that several pachybolid and pseudospirobolellid morphospecies can be interpreted as phylogenetic species as well, particularly within the genus *Tonkinbolus*. Thus we are now preparing a revision and description of six new *Tonkinbolus* species (see e.g. Figs 1-4). The description of another four new species of Pachybolidae (though not from the genus *Tonkinbolus*) from Thailand, Malaysia and Vietnam is in progress as well, while the formal description of new pseudospirobolellid species will be achieved in the course of 2017.

Conversely, the DNA data suggest that several taxa with morphologically very similar sexual organs, nevertheless show strong DNA sequence divergences, indicating that different (phylogenetic) species may be involved (i.e. complexes of cryptic species). However, in order to construct a more comprehensive phylogeny and to discriminate cryptic pachybolid and pseudospirobolellid species, a more complete taxon sampling and more DNA data are still required. This endeavour is part of the future collaboration that the Thai and Belgian partners have set up, and that will entail the further exploration and implementation of high resolution DNA markers (nuclear ribosomal genes, RAD-seq data, mitogenomics), as well as the application of micro CT-scanning on morphological characters.

4. Perspectives for future collaboration between units

The collaboration established between Dr. Piyatida Pimvichai (PP), representing Mahasarakham University and Chulalongkorn University, has a long-term character. The core idea is to consolidate the joint taxonomic and phylogenetic research on millipedes. To that end there will be regular research visits of PP to RBINS and of Thierry Backeljau (TB) to Mahasarakham University. In the same spirit, the joint fieldwork will be continued in the future, with, for example, a first follow-up campaign from 24/7 to 15/8/2016 during which TB will collecting millipedes in southern Thailand with a Thai research team lead by PP. In addition, both PP and TB will propose MSc and PhD thesis subjects at their respective faculties (in case of TB this is at the University of Antwerp), for which both will act jointly as supervisors and which will include student exchange visits between the Belgian and Thai partners. The idea is to formalize this collaboration by means of an MoU and support it by funding from both Belgian and Thai contributions. For example, PP is supported by grants of the Thailand Research Fund, while TB is funded by FWO, EU and RBINS. It is in this context disappointing that BELSPO has removed Thailand from its international research roadmap !

An important aspect in the collaboration between TB and PP, is its extension to other Thai partners at Chulalongkorn University, where PP and TB now team up with the group of Prof. Somsak Panha (SP; Animal Systematics Research Unit, Department of Biology, Chulalongkorn University, Bangkok). This extended collaboration will not only focus on millipede taxonomy, but will also include terrestrial gastropods and Oligochaeta. In this context, TB was appointed as co-supervisor of the PhD work of Parin Jirapatrasilp (PJ; Chulalongkorn University) on the taxonomy and phylogeny of the oligochaete genus *Glyphidrilus* in Thailand. As such, PJ made a first research visit at RBINS from 13/10/2015 to 15/03/2016, and a next research visit is booked for the period November 2016 to March 2017. All this being financed by a scholarship of the Thailand Research Fund.

A third Thai partner involved in this collaboration is Dr. Pongpun Prasankok (PP; Suranaree University of Technology). She is actively involved in the research on millipedes (e.g. joint fieldwork), oligochaetes (e.g. co-supervisor of PJ) and terrestrial gastropods (e.g. data sharing).

At the European side the collaboration between PP and TB is reinforced by Dr. Henrik Enghoff (HE; Natural History Museum of Denmark, Copenhagen), who actively contributes to the descriptive taxonomic work (co-author of joint papers). In the same spirit, collaboration was established with Dr. Didier Vandenspiegel (DVS; Royal Museum for Central Africa, Tervuren, Belgium) and Dr. Sergei Golovatch (SG; Russian Academy of Sciences, Moscow), both providing material and advice, particularly with respect to a new research subject that was started at the end of the fellowship (see point [8] here below).

Finally, as stated before, the collaboration has also been extended to terrestrial gastropods. As such, the joint fieldwork is more effective as it serves several taxonomic groups. The work on terrestrial gastropods has already produced interesting results that will be presented as a joint poster (and abstract) at the 'World Congress of Malacology 2016, Penang, Malaysia, 18-24 July 2016' (see Attachment 3). The research presented in this poster is based on material collected during the fieldwork in Thailand (20/08-07/09/2014) organized by PP and is currently being summarized in a manuscript.

Against this background, the immediate, in concreto research perspectives of the collaboration that was established by the BELSPO fellowship to PP are as follows:

[1] Finishing and publishing the morphological descriptions of the new pachybolid and pseudospirobolellid species characterized during the fellowship, by PP, TB, HE and SP.

[2] Publishing the results of the DNA barcoding, species delimitation, and phylogenetic analyses (including an evolutionary phenotypic character analysis by tracing phenotypic character state changes on the phylogenetic trees) conducted during the fellowship, by PP, TB, HE and SP.

[3] Finalizing and publishing the RAD-seq analyses and mitogenomic data, by PP, TB, HE and SP.

[4] Joint poster presentation at the 'World Congress of Malacology 2016, Penang, Malaysia, 18-24 July 2016', and subsequent publishing of these results, by PP, TB and SP (see Attachment 3).

[5] Organizing joint fieldwork from 24/7 to 15/8/2016, by PP, TB and PP.

[6] Second research visit to RBINS, by PJ to TB (November 2016 – March 2017).

[7] Joint participation of to the '17th International Congress of Myriapodology, Krabi, Thailand, 23-26 July 2017' with at least one joint oral contribution and one joint poster, by PP, TB, HE and SP.

[8] Spring 2017: Completing a challenging side research project on the phylogeny and phylogeography of the small, endemic African millipede family Ammodesmidae, which comprises only two, geographically disjunct genera. PP has established already COI and 16S rDNA sequences of some old museum samples of representatives of this family. This work is done by PP, TB, SP, DVS and SG.

[9] Further testing of nuclear ribosomal genes (18S rDNA, ITS1, ITS2,…) as potential phylogenetic and taxonomic markers in millipede taxonomy, by PP, TB, HE and SP.

On the longer run PP will visit RBINS again in the second half of 2017 to proceed with the application of RAD-seq and to explore the possibilities of micro CT-scanning for the morphological characterization of the gonopods and vulvae.

5. Valorisation/Diffusion (including Publications, Conferences, Seminars, Missions abroad...

[1] Participation to the '16th International Congress of Myriapodology, Olomouc, Czech Republic, 20-25 July 2014', with the oral presentation "Systematics of the millipede family Pachybolidae, with emphasis on the Southeast Asiatic Fauna" (abstract book p. 70; see Attachment 1). Financed by RBINS.

[2] Participation to 'Variant Discovery in Next-Generation Sequencing (NGS) Data' Workshop, 24-26 June 2014. Financed by RBINS.

[3] Organization of fieldwork in Southern Thailand (20 August to 7 September 2014) to collect millipedes. Financed by The Thailand Research Fund and RBINS.

[4] Research visit to the Naturhistorisches Museum, Vienna, Austria, 20-24 July 2015 to study the collections of Carl Graff Attems. Financed by Synthesys.

[5] Participation to the DEST (Distributed European School of Taxonomy) training course 'Philosophy of Biological Systematics', RBINS, Brussels, 7-11 September 2015. Financed by RBINS

[6] Presenting the research seminar 'Systematics of the Cylindrical millipede family Pachybolidae with emphasis on the Southeast Asiatic fauna' at RBINS, 29 October 2015.

[7] Research visit to the Natural History Museum of Denmark, 20-30 January 2016 to study the holdings in this museum and to discuss pachybolid taxonomy with Dr. Henrik Enghoff, with whom a permanent collaboration is maintained. Financed by Synthesys.

[8] Publication (see also Attachment 2):

Pimvichai, P., Enghoff, H., Panha, S. and Backeljau, T. 2016. A revision of the *Thyropygus allevatus* group. Part V: Nine new species of the extended *opinatus* subgroup, based on morphological and DNA sequence data (Diplopoda: Spirostreptida: Harpagophoridae). - *European Journal of Taxonomy*, 199: 1-37. http://dx.doi.org/10.5852/ejt.2016.199

6. Skills/Added value transferred to home institution abroad

The major new skills that Dr. Pimvichai picked up during her fellowship were:

[1] The practice of preparing DNA libraries for 'Restriction Site Associated DNA sequencing' (RAD-seq), a Next Generation DNA Sequencing methodology that can be applied to non-model taxa for which there is little or no beforehand genomic information, and which therefore is very suitable for the genomic analysis of millipedes.

[2] Processing of RAD-seq sequences using the PyRAD pipeline. This is essentially a 'big data' approach, allowing to handle the hundreds of thousands to millions of sequence reads produced by RAD-seq, yielding concatenated sequences of 500,000 to over 2,000,000 bp long and containing several tens of thousands polymorphic (informative) single nucleotide positions (SNP).

[3] The practice of preparing DNA samples for complete mitochondrial DNA sequencing (mitogenomics) and the subsequent analysis of complete mitochondrial genomes.

[4] The application of different species delimitation methods to detect DNA barcoding gaps and taxonomic heterogeneities in DNA sequence data.

[5] Microphotography of genital organs using stacking procedures.

These skills will be further implemented in Dr. Pimvichai's lab in Mahasarakham University and in the associated lab at Chulalongkorn University.