MANUSCRIPT EVALUATION FORM

DNA barcodes and light spectra of nine sympatric fireflies in northern Taiwan

King-Siang Goh[1], Jing-Han Ni[2], Tzi-Yuan Wang[3]*

Reviewer's recommendations:

X Accept with MAJOR revision

Reviewer's comments

Considerable work has been done, is new, and worthy of publication.

However, the paper shows little understanding of the biology of the group, has not referenced many similar studies, has no discussion, few and inaccurate comparisons to previous studies, and the most unusual conclusions! I have spent over a week on this, and have had enough, and have now stopped. Details below. Comments, suggestions, and corrections are included on PDF supplied.

As it is currently, the paper is insubstantial because:

- description of methods are minimal require more detail
- discussion and conclusions are inadequate as they are currently a summary of results
- insufficient and unsatisfactory discussion based on evidence from results
- no acknowledgement of weakness of data or results that compromise ability to make well-supported inferences
- few, inaccurate, and incomplete comparisons of results with previous scientific work:

Eg introduction paragraph 2:

The molecular phylogeny of Lampyridae was recently reassessed (Chen et al. 2019; Martin et al. 2017; Stanger-Hall et al. 2007), and it identified the following monophyletic groups: Ototretinae, Cyphonocerinae, Luciolinae (incl. Pristolycus), Amydetinae, "cheguevarinae" sensu Jeng 2008, Photurinae, and Lampyrinae (with the exception of four polyphyletic taxa).

"cheguevarinae" was used in Martin et al. 2017.

Which paper cited does 'it' refer to? 'it' appears to refer to Martin et al. 2017 as the summary of 'monophyletic groups' given here is almost exactly the same as in the abstract for that paper.

Also missing from this list is Martin et al. 2019. Admittedly that is a very recent paper, but it was published at the same time as Chen et al. 2019, that is cited.

Is the paper a new contribution to the topic? YES, and unfortunately the 'new' work is not highlighted.

Are the methods adequate and sufficiently well described? Adequate just, more details would be appreciated though. Methods should have used methods previously used or explained how they differed and why

Have the appropriate statistical analyses been performed? Not well explained

Is the information presented in a logical sequence and concise manner? Not completely, some sections need to be moved (as noted on MS), and in areas too concise. Background/introduction should have outlined what was already known for – gender differences, population differences, species differences for - measurements; light intensity, light spectra, COI

This paper needs (possibly in the introduction, if not, in the relevant parts of the discussion) an outline of the biology of fireflies that links the disparate studies included
1- light spectra, light intensity, mating behaviour; mate, species, and generic recognition
2 - morphology, DNA, relationships

Are the illustrations necessary and satisfactory? YES, although the phylogenies are small. They need to be printed full page to be readable.

Are the tables necessary and satisfactory? Yes, although would appreciate Table of all sequences used with Genbank #, and references. Also link to alignment would be appreciated.

Is the nomenclature satisfactory and consistent with The Code? YES

Is the paper correct with respect to the issues and literature involved? No, inadequate background information especially biology, and insufficient comparison with previous work to place the importance of this work into a scientific context.

Many references to previous work not included.

Are the interpretations and conclusions justified and adequate? No. Discussions are often a repeat of results with, few and sometimes inaccurate comparisons to other studies, no acknowledgement of limitations and very little discussion. Discussions should have compared results with what found before, and suggested hypotheses to explain results especially where they differed from previous studies, and placed their study of Taiwanese fireflies into a world/Asian context. Conclusions are poorly explained, not based on results, and sometimes irrelevant.
Conclusions should have summarised their discussion, highlighting significant findings.

Other issues and comments below in detail, and directly on the MS, but not anywhere near all..

Do you wish to remain anonymous? No - I would appreciate acknowledgement by name and institution for my review. Christine Lambkin, Queensland Museum
If any questions arise from my review, I encourage communication with the authors.

Christine Lambkin, Queensland Museum 10/8/2020

Taxonomy, Identification, Morphology, Biology

When including the **author of a taxonomic name**, if you also include the year of publication, you should add the paper to the References. Unfortunately, adding to the references suggests that the relevant paper has been read, which is probably not true. Best to remove all years of publication from taxonomic names.
Taxonomic name authors continue to be used throughout, not only at first mention in the text. Remove following first use in text.

Please note that Luciola substriata in the phylogeny is now Sclerotia substriata see Ballantyne et al. 2019.

How were the specimens identified to species? Using keys? Which ones? With reference to a well identified collection? Verified by an expert? Who? Especially the females and larvae, which can be very difficult to identify. How were males and females associated? This should all be outlined in the methods.

Where are the specimens deposited?

Morphology
Measurements of body length an issue – As stated in Jusoh et al. 2018
Body length is an artificial representation consisting of median length of pronotum plus length of elytra; this will usually appear longer than the actual specimen as in pinned specimens the pronotum droops. The head is not included because it also may droop, or be variously retracted within the prothoracic cavity.
More details of where these measurements were taken should be included.

Absolute measurements rather than ratios may be an issue as adult size may be affected by amount of available larval food.

Morphological measurements
Results. conclusion in abstracts that makes little sense – not, or poorly explained in MS discussion
Four of the species had significantly different characters between females and males: the front wing width of *Abscondita chinensis* (Linnaeus, 1767), pronotum width of *Abscondita cerata* (Olivier, 1911), pronotum length of *Aquatica ficta* (Olivier, 1909), and body length of *Luciola curtithorax* Pic, 1928.
With P. praetexta only collected as larvae and no males for C. costipennis and no females for L. filiformis, these comparisons are only for 6 species. And you find 'significant differences' between the males and females for 4 of the 6 species.

Significant differences were calculated. What is the null hypothesis, that there are no differences between males and females? of the same species? No comparisons between species?
At what level of significance? The levels at which statistical significance has been reached are unclear. They seem to vary in Table 4.
Also what is the effect of such small numbers for many species?

These limitations should be outlined and their affect on the inferences you can make should be addressed in the text.

Comparisons of male and female measurements

Has sexual dimorphism in size been reported before in this group? If so, where, reference, and compare your findings. Or is it so common that it is never mentioned.
Fu in the Ballantyne et al. 2013 paper reports all the morphological measurements taken here, for Abs. chinensis in a much more comprehensive manner. Comparisons to that work are not made in this paper.

Some female fireflies don't fly – including Luciola filiformis – but only males collected here, possibly for that reason. Again that limitation is not discussed.

There are however many papers examining the size differences in fireflies related to their biology, especially the big differences in some of the Lampyrinae e.g. Pyrocoelia, Diaphanes where the females are flightless.
Ballantyne pers. com. 'The whole idea of the change to flightless females being so large is that they accumulate more food in the larval stage and, this bit is important, as they are not supposed to rely so much on the nutrients they would derive from a spermatophore. So these big Lampyrinae females do not receive spermatophores. And the flighted Luciolinae produces spermatophores. We do not know about the flightless ones but the prediction is that they do but that they are on the path towards losing spermatophores. So adult size is affected by the larval intake conspicuously in the Lampyrinae flightless females, but it is a bit of a circular argument.

Biology

The biology of communication with light flash patterns in fireflies is well outlined in Stanger-Hall & Lloyd 2015
When observing fireflies, it is possible to find oneself in the presence of 10 or more flashing species (Lloyd 1969a), all active in the same habitat and looking for a conspecific mate. Males and females use species-specific light signals as an interactive morse-code that identifies the species and the sex of (Lloyd 1966). Flashing males tend to be airborne and searching for sedentary females. Females, with rare known exceptions, remain perched in the vegetation below the male activity space. Upon recognition of the correct signal, females respond to male flashes with a species-specific response delay (Lloyd 1966). Males will use this delay when deciding whether to continue signalling to a particular female, or whether to continue their search flight. If females respond with the correct delay, the pair will continue this dialogue as the male approaches to physical contact. In some species males may compete with conspecific males by interfering in their signal dialogue with the female (e.g., P. macdermotti: Lloyd 1983), or by scramble competition (e.g., P. pyralis: and P. carolinus: Faust 2010). When females mistakenly respond to a male of another species, the signaling male either turns away immediately due to the inappropriate delay of the response, or flashes back and forth a few times before leaving (Lloyd1968). female, but usually recognize their Lloyd 1966), possibly mediated by cuticular (Higgie et al. 2000; Ming and Lewis 2010, 2008).
There is nothing similar in this paper and it is needed to place the study into context, and to explain the results in the discussion, and infer conclusions.

What is light for in fireflies – for the males to attract females? Why do males have stronger light than females? Ballantyne pers. com. 'the perceived wisdom is that the males flash to attract a female and

that once she has made her choice she flashes in response. It is sort of the female choice. They fly about flashing like mad depending on the numbers of them but females are much more choosy and may not fly in these displays at all but just shine their light from the ground. So the males are trying to attract a response from a female not to find her.'

Endemic is used incorrectly throughout, I believe. Endemic means native and restricted to a certain place. Therefore some species may be endemic to Taiwan, or to a particular area of Taiwan, and not found anywhere else. Here I believe endemic is used to refer to species found in both localities investigated. Please clarify and correct if necessary.

Sympatric species is used incorrectly throughout, I believe. Sympatric species are found in the same locality at the same time
8 species (possibly 9 if curtithorax is included see below) are found at Nanzhuang
5 species (6 if curtithorax is included) are sympatric at Nankang
There is no evidence presented here that those species were present at the same time or even day, in each of those localities
Unfortunately Table 4 indicates that you measured the light spectrum of a single male curtithorax from Nanzhuang, as does your raw data. Please check, and correct throughout if necessary.

DNA, phylogenies & relationships
DNA sequences – males and females? How many didn't work?
Some of your sequences appear to be of multiple specimens with the same Genbank # - what did you deposit in Genbank, a consensus? These details should be included in methods.
Multiple sequences for the same species should be noted in the text.
How many are newly sequenced here? I think 3 – C. Sauteri, Abs. cerata, L. kagiana. This should be highlighted in the study, and their placement in the phylogeny discussed in detail.

No BLAST check for contamination

Molecular phylogeny
The use of COI alone in phylogenetic analyses to determine species and higher-level relationships is controversial and generally not well accepted in the scientific community. Please clarify that you understand the issues, with references, and outline in the paper the consequent limitations on inferences from your analyses.
COI has been used in Lampyrid studies to align males and females, and examine population vs species separation, – see:

Jusoh, W.F.A., Ballantyne, L., Lambkin, C.L., Hashim, N.R. & Wahlberg, N. (2018) The firefly genus *Pteroptyx* Olivier revisited (Coleoptera: Lampyridae: Luciolinae). *Zootaxa*, 4456 (1), 1–71. https://doi.org/10.11646/zootaxa.4456.1.1

Jusoh, W.F.A., Hashim, N.R., Sääksjärvi, I., Adam, N.A. & Wahlberg, N. (2014) Species delineation of Malaysian Mangrove Fireflies (Coleoptera: Lampyridae) using DNA barcodes. *The Coleopterists Bulletin*, 68 (4), 703−711. https://doi.org/10.1649/0010-065X-68.4.703

The latter paper, especially, overcomes many of the limitations of your paper with more comprehensive explanation of methods, outlines and discusses clearly the limitations on inferences where data is incomplete, and discusses results with comparisons to previous studies.

The COI sequences of closely-related species were downloaded from GenBank. There were a total of 520 positions and 80 nucleotide sequences in the final dataset.
Were there other specimens from Genbank of the 9 species sequenced? There are complete miochondrial genomes for Abs. chinensis & L. curtithorax species available in Genbank from Wang & Fu 2018 & Hu & Fu 2018. Why were they not included?

There are COI sequences for other Asian fireflies including
Curtos okinawanus (Muraji et al 2012 & Martin et al. 2017),
Abs. terminalis (Liu et al. 2017),
and species of Colophotia Motschulsky, Poluninius Ballantyne, and Pyrocoelia Gorham, and many species of Pteroptyx Olivier (Jusoh et al. 2014);
and complete miochondrial genomes for Abs. anceyi (Hu & Fu 2018) available in Genbank.
Why were they not included? Please explain.

No table outlining all sequences used and where they came from...

No discussion of issues with alignment
No alignment presented, or made available

Molecular phylogeny inferred by DNA barcodes
The NJ (Figure 4) and ML trees (Figure 5) indicate that the genera *Abscondita*, *Curtos*, *Aquatica,* and *Luciola* belong to Luciolinae, while *Pyrocoelia* belongs to Lampyrinae.
This is not only inadequate it is inaccurate. The NJ and ML trees differ significantly in regards to the placement of the genera Abscondita and Curtos; the Luciolinae is not monophyletic; Rhagophthalmus (Rhagophthalmidae) moves around always causing issues. See below.

In the NJ tree (Fig. 4)
The species sequenced here are generally placed correctly with congeners or conspecifics

- although L. filiformis does not form a clade with the other sequence of L. filiformis, rather both forming a sister arrangement to L. parvula;
- and Aq. ficta here forms a sister relationship to the other sequence of Aq. ficta and Aq. leii

Luciolinae is paraphyletic as:

- Curtos is separated from the remaining Luciolinae by Stenocladius that Janisova & Bocakove 2013 moved to the Ototretinae, tentatively supported by Martin et al 2019. Stenocladius is not in the Luciolinae. Chen et al. 2019 considered *Stenocladius* should be placed into the subfamily Ototretadrilinae with *Ototretadrilus*.
- Rhagophthalmus (Rhagophthalmidae) is placed within the Luciolinae

Pristolycus (separate tribe of Luciolinae) is placed as sister to Sclerotia (Luciolini)
Pyrocoelia (in the Lampyrinae) is rendered paraphyletic by Lampyris
Photurinae renders Lampyrinae paraphyletic

In the ML tree (Fig. 5)
The species sequenced here are generally placed correctly with congeners or conspecifics including L. filiformis forming a clade with the other sequence of L. filiformis

- Aq. ficta here continues to form a sister relationship to the other sequence of Aq. ficta and Aq. leii

Luciolinae is now polyphyletic as:

- Curtos forms a clade, sister to the Ototretinae completely separated from the remaining Luciolinae
- Absconditus species sequenced here form a clade with Rhagophthalmus (Rhagophthalmidae), Pterotinae, Cyphonoceriinae sister to Phausis (Lampyrinae) sister to the Luciolinae

Pristolycus (separate tribe of Luciolinae) remains sister to Sclerotia (Luciolini)
Pyrocoelia (in the Lampyrinae) remains rendered paraphyletic by Lampyris
Photurinae forms an unresolved polytomy with the Lampyrinae, and with Stenocladius (Ototretinae or Ototretadrilinae)

Different papers present different phylogenies, even differ in same paper, as here. Need much better discussion on how their phylogeny compares to other studies.

Results. conclusion in abstracts that makes little sense – not, or poorly explained in MS discussion
The COI barcode suggests a high resolution of species identification and a phylogeny that is consistent with previous mitogenomic phylogenies. Incomplete and inaccurate. See above.

Light spectra and intensity

No attempt to separate species via flashing patterns in the field

Measurement of light intensity – placing probe against the light organ. Does that affect the light emission?

The following paper examined emission spectra and flashing patterns of Abscondita chinensis (included in this study) and was not referred to in this paper, for that aspect of the study.

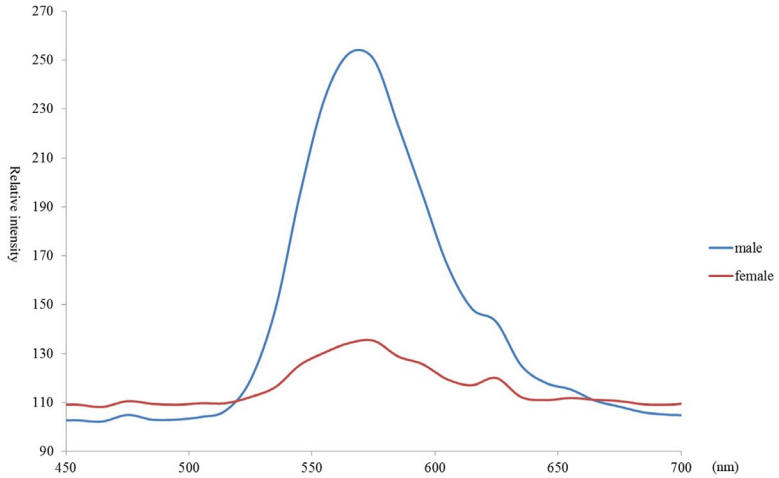
Ballantyne L, Fu XH, Lambkin C, Jeng ML, Faust L, Wijekoon WMCD, Li DQ, and Zhu TF. 2013. Studies on South-east Asian fireflies: *Abscondita*, a new genus with details of life history, flashing patterns and behaviour of *Abs. chinensis* (L.) and *Abs. terminalis* (Olivier) (Coleoptera: Lampyridae: Luciolinae). *Zootaxa* 3721:1-48.

This is how that paper describes the method for capturing light spectra data:

Emission spectra. The colour of luminescent flashes was measured in both males and females in the laboratory using an Ocean Optic USB4000 spectrometer (Ocean Optics Inc., Dunedin, Florida, USA). The reflectance reading (250–700 nm) was recorded from a circular spot (diameter 2 mm) on the sample (ventral abdominal lanterns of fireflies), perpendicular to and 3 mm above the lanterns. Five readings were taken for each firefly when it emitted flashes.

Eleven fireflies (6 females and 5 males) were collected by Fu either in Mt Da bei or Red Flag Village, and transported back to the laboratory for measurements. Thirty readings of females and 25 of males, held by soft forceps, were recorded at 25°C and 75% humidity. The mean emission spectra of these readings were calculated for each male and female firefly.

Fu reports for Abs. chinensis - The bioluminescence emission of both the sexes is yellow (λmax=565nm) (Fig. 17).



There is no statement anywhere in this paper regarding the actual colour of the bioluminescence.

That same 2013 paper in the discussion refers to Fu's findings in this manner:

Biology and light patterns. Once collected, it is difficult to distinguish between Abs. chinensis and Abs. terminalis because of their similarity in colour and size. Male Abs. chinensis courting flash in flight appears as a multi-pulse flash train to unaided eyes, but actually consists of modulated ca 1 sec single flash of varying intensity when recorded and interpreted with photo-intensifiers and software. Male Abs. terminalis have rapid yet distinct ca 1 sec multi-pulse flash trains with an interval of about 1.5 seconds while the interval for Abs. chinensis is much longer (about 2.8 seconds). For a trained observer, especially when in the preferred habitat of Abs. chinensis (forest) and Abs. terminalis (open fields and paddies) it is possible to distinguish these two species by male flash patterns.

Flash exchanges between male and female fireflies have long been known to bring the sexes together for mating (McDermott 1911; Lloyd 1966, 1972; Ohba 2004; Vencl 2004; Fu et al. 2006). Some species of the genus Luciola have simple question-and-answer dialogue while others have sedentary females that broadcast their presence before responding to a particular male (Kaufmann 1965). Abs. terminalis has a special courtship protocol. Patrolling males produce species-specific multi-pulse flashes; yet the female, rather than responding with a fixed delay, instead broadcasts her location independently. Courting males landed very close to these broadcasting females and mounted directly without further flash dialogues. After peak courtship patrolling each evening, many males perched on the grass tips, producing slow single pulse flashes via one segment, V6, followed by several fast bright single pulses flashes from both V6 and 7. The function of these unusual perching flash behaviours remains unknown.

There is nothing similar in this paper and it is needed to place the study into context, and to explain the results in the discussion, and infer conclusions.

Other studies of light intensity?

Other studies of light spectra?

Comparison in this paper with other studies is minimal.

Results. conclusion in abstracts that makes little sense – not, or poorly explained in MS discussion

Their light spectra, the λ_{max} of which was 552– 572 nm, may be key for most sympatric fireflies to identify one another in the dark. How do these two statements relate?
Never clear as to which species are sympatric ie found at the one locality at the same time.

In discussion
Nevertheless, most sympatric fireflies could distinguish between each other based on the light spectra of their flashes (Stanger-Hall & Lloyd 2015).
Stanger-Hall & Lloyd 2015 cited:
Animal communication is an intriguing topic in evolutionary biology. In this comprehensive study of visual signal evolution, we used a phylogenetic approach to study the evolution of the flash communication system of North American fireflies. The North American firefly genus *Photinus* contains 35 described species with simple ON–OFF visual signals, and information on habitat types, sympatric congeners, and predators. This makes them an ideal study system to test hypotheses on the evolution of male and female visual signal traits. Our analysis of 34 *Photinus* species suggests two temporal pattern generators: one for flash duration and one for flash intervals. Reproductive character displacement was a main factor for signal divergence in male flash duration among sympatric *Photinus* species. Male flash pattern intervals (i.e., the duration of the dark periods between signals) were positively correlated with the number of sympatric *Photuris* fireflies, which include predators of *Photinus* . Females of different *Photinus* species differ in their response preferences to male traits. As in other communication systems, firefly male sexual signals seem to be a compromise between optimizing mating success (sexual selection) and minimizing predation risk (natural selection). An integrative model for *Photinus* signal evolution is proposed.
Stanger-Hall & Lloyd 2015 does not appear to me to be relating light spectra to distinguishing conspecifics or congeners. It examined mainly flash duration and flash intervals within one genus.

Results. conclusion in abstracts that makes little sense – not, or poorly explained in MS discussion
Furthermore, the maximum light intensity of males is 1.3–11-fold higher than that of females. This implies that they can socialize at up to 3.3 meters apart. How? How do these two statements relate?

Understanding of behaviour not clear – Who is finding who? If males are finding females then it comes down to whether the males can detect the females at their lower light intensity.

The average light intensity of their light organs ranged from 0.7–4 lux in females and 1.3–3.5 lux in males, which suggests that fireflies start flashing when the environmental light intensity is 4.69–7.63 lux.
The two parts of this sentence do not relate. The difference in average light intensity between genders has nothing to do with the environmental light intensity levels at which they start to flash.

Conclusions includes:
This study establishes the light spectrum and intensity of nine sympatric fireflies, and can be referenced to ensure that light pollution in habitats does not become high enough to disrupt firefly mating.
I think you are trying to imply that environmental light intensity must remain below 4.69 lux, the lowest level at which you report that they flash. Unfortunately you never make that link in this paper.

References
Cited References all included – except Jeng 2008, which is an understandable miss.

Many taxon names are not italicised.
Also several articles have titles with all words capitalised.

introduction paragraph 1:
'Moreover, the larvae are used as biological controls in some species, such as *Pyrocoelia atripennis*, which eats invasive snails (Sato 2019).'
Incorrect reference -The Sato reference did not even suggest the larvae could be used in biocontrol - this one did
Fu X, Meyer-Rochow VB. 2013. Larvae of the firefly Pyrocoelia pectoralis (Coleoptera: Lampyridae) as possible biological agents to control the land snail Bradybaena ravida. Biological Control 65(2):176–183 DOI 10.1016/j.biocontrol.2013.02.005.
Please check and correct if necessary.

However, I consider **some other papers should have been cited,** discussed, and included in the references, especially:

Jusoh, W.F.A., Ballantyne, L., Lambkin, C.L., Hashim, N.R. & Wahlberg, N. (2018) The firefly genus *Pteroptyx* Olivier revisited (Coleoptera: Lampyridae: Luciolinae). *Zootaxa*, 4456 (1), 1–71. https://doi.org/10.11646/zootaxa.4456.1.1

Jusoh, W.F.A., Hashim, N.R., Sääksjärvi, I., Adam, N.A. & Wahlberg, N. (2014) Species delineation of Malaysian Mangrove Fireflies (Coleoptera: Lampyridae) using DNA barcodes. *The Coleopterists Bulletin*, 68 (4), 703−711. https://doi.org/10.1649/0010-065X-68.4.703

Martin et al. 2019.
Ballantyne et al. 2019.

DNA barcodes and light spectra of nine sympatric fireflies in northern Taiwan (#49327)

First submission

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Please submit by **10 Aug 2020** for the benefit of the authors (and your $200 publishing discount) .

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Have you read the author notes on the guidance page?

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Structure your review

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3. **VALIDITY OF THE FINDINGS**
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- Raw data supplied (see PeerJ policy).

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- Original primary research within Scope of the journal.
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- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- All underlying data have been provided; they are robust, statistically sound, & controlled.
- Speculation is welcome, but should be identified as such.
- Conclusions are well stated, linked to original research question & limited to supporting results.

Standout reviewing tips

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Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
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Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

DNA barcodes and light spectra of nine sympatric fireflies in northern Taiwan

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Background. Fireflies are well-known not only for their ecological and cultural value, but also for their bioluminescent mechanism and evolutionary role. Taiwan has 56 firefly species that are recognized based on morphology, but studies on their genetic and biologic characters remain scarce or nonexistent.

Methods. In this study, DNA barcodes and bioluminescent properties from nine Taiwanese species were investigated. These fireflies, including four endemic species, were collected from two sampling locations in northern Taiwan from April to August. The specimens were photographed and their morphological measurements taken, their light spectra and light intensities were recorded, and their cytochrome oxidase I (COI) gene was used as a DNA barcode to reveal their phylogenetic relationships.

Results. Four of the species had significantly different characters between females and males: the front wing width of *Abscondita chinensis* (Linnaeus, 1767), pronotum width of *Abscondita cerata* (Olivier, 1911), pronotum length of *Aquatica ficta* (Olivier, 1909), and body length of *Luciola curtithorax* Pic, 1928. Their light spectra, the λ_{max} of which was 552–572 nm, may be key for most sympatric fireflies to identify one another in the dark. The average light intensity of their light organs ranged from 0.7–4 lux in females and 1.3–3.5 lux in males, which suggests that fireflies start flashing when the environmental light intensity is 4.69–7.63 lux. Furthermore, the maximum light intensity of males is 1.3–11-fold higher than that of females. This implies that they can socialize at up to 3.3 meters apart. The COI barcode suggests a high resolution of species identification and a phylogeny that is consistent with previous mitogenomic phylogenies

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Abstract

Background. Fireflies are well-known not only for their ecological and cultural value, but also for their bioluminescent mechanism and evolutionary role. Taiwan has 56 firefly species that are recognized based on morphology, but studies on their genetic and biologic characters remain scarce or nonexistent.

Methods. In this study, DNA barcodes and bioluminescent properties from nine Taiwanese species were investigated. These fireflies, including four endemic species, were collected from two sampling locations in northern Taiwan from April to August. The specimens were photographed and their morphological measurements taken, their light spectra and light intensities were recorded, and their cytochrome oxidase I (COI) gene was used as a DNA barcode to reveal their phylogenetic relationships.

Results. Four of the species had significantly different characters between females and males: the front wing width of *Abscondita chinensis* (Linnaeus, 1767), pronotum width of *Abscondita cerata* (Olivier, 1911), pronotum length of *Aquatica ficta* (Olivier, 1909), and body length of *Luciola curtithorax* Pic, 1928. Their light spectra, the λ_{max} of which was 552–572 nm, may be key for most sympatric fireflies to identify one another in the dark. The average light intensity ~~of~~ their light organs ranged from 0.7–4 lux in females and 1.3–3.5 lux in males, which suggests that fireflies start flashing when the environmental light intensity is 4.69–7.63 lux. Furthermore, the maximum light intensity of males is 1.3–11-fold higher than that of females. This implies that they can socialize at up to 3.3 meters apart. The COI barcode suggests a high resolution of species identification and a phylogeny that is consistent with previous mitogenomic phylogenies

Introduction

Among terrestrial bioluminescent insects, fireflies (Lampyridae) have the most charismatic shine, using their glow for mating or as aposematic signals at night (Oba et al. 2011). Fireflies in Coleoptera are also the most diverse terrestrial group of bioluminescent organisms. Firefly life history and bioluminescence have been studied over a century and have offered bioinspiration for many inventions and methods, such as a method for detecting gene expression (biomedical), improvements in LED technology (industrial), and some algorithms (mathematical) (Kaskova et al. 2016; Kim et al. 2016; Yang 2009). Fireflies are also considered an environmental index species for assessing light, water, and soil pollution. Moreover, the larvae are used as biological controls in some species, such as *Pyrocoelia atripennis*, which eats invasive snails (Sato 2019). Firefly population sizes are dramatically affected by changes in land-use, as habitat deterioration and artificial night lighting decrease their populations (Firebaugh & Haynes 2016; Owens et al. 2018).

Over 2,100 firefly species have been reported and are widely distributed in temperate and tropical regions, including Eurasia, America, New Zealand, and Australia. Fifty-six species have been described from Taiwan to date, but very few reports have been made on their biodiversity, ecological habitats, comparative morphology, life cycle, or behavior (Ballantyne et al. 2013; Ballantyne et al. 2015; Goh & Li 2011; Ho et al. 2010; South et al. 2008). The molecular phylogeny of Lampyridae was recently reassessed (Chen et al. 2019; Martin et al. 2017; Stanger-Hall et al. 2007), and it identified the following monophyletic groups: Ototretinae, Cyphonocerinae, Luciolinae (incl. Pristolycus), Amydetinae, "cheguevarinae" sensu Jeng 2008, Photurinae, and Lampyrinae (with the exception of four polyphyletic taxa). However, DNA barcoding of Taiwanese fireflies and systematic studies of their light spectrum/intensity remain scattered. Therefore, this study investigated nine species in northern Taiwan to establish basic information for further applications.

Materials & Methods

Specimen collection and habitat

141 specimens of nine species were collected using hand dip nets from two habitats in suburbs of Taipei, Taiwan—Nankang (25°01'40.4"N 121°38'02.6"E) and Miaoli County, Nanzhuang (24°37'53.5"N 121°01'37.0"E)—at 18:30–19:30 from April to August, 2017 (Fig. 1, Table 1). The habitat temperature, relative humidity, and light intensity (lux) were measured with HOBO U12-012 data loggers.

Light spectrum/intensity measurement

The wavelength (λ_{max}) and light intensity (nW/cm²) of living samples were measured by USB2000+ spectrometer (Ocean Optics) and PD300 power meter (Ophir), respectively. The wavelength and light intensity measurements were performed in a dark room by directly attaching the detector of the USB2000+ spectrometer or PD300 to the light organ of a trapped firefly. The average wavelength peak and λ_{max} were obtained from the average of 3~5 measurements. The light intensity of the flash was obtained by averaging each flash from 3~10 min of recording data. For DNA extraction, several specimens were then stored at -80°C. The remaining specimens were stored at -20°C for morphological measurement. To compare the light intensity data from PD300 and HOBO U12-012 using the same units, all data in the energy unit nW/cm² were converted into lux via the conversion 1 lux = 1.46412884333821E-07 W/cm² (at 555 nm).

Morphological measurements

Five ~~major~~ characters (body length, pronotum length, pronotum width, front wing length, and front wing width) were measured using a dissecting microscope and a micrometric ruler. Significant differences between females and males were calculated for each character by Student's t-test and *F*-test. * denotes p-value < 0.05, ** denotes p-value < 0.01, *** denotes p-value < 0.001.

DNA barcode sequencing

Crude DNA was extracted from thorax muscles via the ZR Tissue & Insect DNA MicroPrep™ kit (D6015). Two universal primers (ClepFolF 5'-ATTCAACCAATCATAAAGATATTGG-3' and ClepFolR 5'-TAAACTTCTGGATGTCCAAAAAATCA-3') were designed based on the comprehensive DNA barcode database of beetles (Hendrich et al. 2015) to amplify a 620-bp segment including the cytochrome oxidase I (COI) gene. Polymerase chain reactions (PCRs) in 50-μL volumes were performed with a dNTP concentration of 200 μM and primer concentration of 0.3 μM, with 50 ng of genomic DNA, one unit of TaKaRa Taq™ DNA Polymerase, and the buffer supplied by the manufacture. The PCR was run for 35–40 cycles under the following conditions: denaturation at 95°C for 30 s, annealing at 50~55°C for 40 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The product mixture was used as a template for DNA sequencing. Sequences were deposited in GenBank under accession numbers MT534191-MT534201 (Table 2).

Molecular phylogeny

The COI sequences of closely-related species were downloaded from GenBank. There were a total of 520 positions and 80 nucleotide sequences in the final dataset. Sequences were then aligned using the ClustalX program (Thompson et al. 2002) with manual modifications. Neighbor-joining (NJ) (Saitou & Nei 1987) and maximum-likelihood (ML) trees were constructed using GTR+G+I distances in MEGA7 (Kumar et al. 2016) with 500 bootstrap replications (Felsenstein 1985). The substitution model (parameter) used to calculate GTR+G+I distances (Nei & Kumar 2000) was selected using Modeltest v3.7 (Posada & Crandall 1998). The differences in the composition bias among sequences were considered in the evolutionary comparisons (Tamura & Kumar 2002).

Results

Habitat environment for firefly activity

During the firefly mating season, fireflies became active at around 18:30. To explore the environmental factors that may have triggered firefly flashing, the change in environmental conditions before and after fireflies began flashing and/or flying were investigated. The temperature, relative humidity, and light intensity of the firefly habitat were recorded at twilight (18:00~18:30) and the period that the fireflies began flashing/flying (18:30~18:45) using HOBO U12-012 data loggers (Table 3). The average environmental light intensity around twilight was 58.6–390.8 lux, while the flashing/flying began at an average environmental light intensity of 4.69–7.63 lux. Most fireflies, especially the dominant *Abscondita cerata* (Olivier, 1911), are highly active from 18:30–19:30. The average temperature ranged from 17.1–25.0°C. The average relative humidity ranged from 71.2–95.8%. The evenings after sunny days with high humidity and cool temperature are the most suitable for firefly activity.

Species identification

We collected individuals of nine adult species from two habitats in northern Taiwan (Table 1 and Figure 2). Eight of these species were found in Nanzhuang and only five were found in Nankang. Five species—*Aquatica ficta* (Olivier, 1909), *Luciola filiformis* Olivier, 1913, *Abscondita cerata* (Olivier, 1911), *Luciola kagiana* Matsumura, 1928, and *Pyrocoelia praetexta* Olivier, 1911—were found in both habitats.

During our investigation, *Luciola curtithorax* Pic, 1928 was only found in Nankang and *Abscondita chinensis* (Linnaeus, 1767), *Curtos sauteri* Olivier, 1913, and *Curtos costipennis* (Gorham, 1880) were discovered only in Nanzhuang. Among the collected species, *A. cerata*, *C. sauteri*, *L. kagiana,* and *P. praetexta* are ~~endemic~~ (to both habitats), and *C. costipennis* is less common than the other species.

Sexual dimorphism

Table 4 shows the differences in five major characters between females and males; the following were significantly different: the front wing width of *Abscondita chinensis* (Linnaeus, 1767), pronotum width of *Abscondita cerata* (Olivier, 1911), pronotum length of *Aquatica ficta* (Olivier, 1909), and body length of *Luciola curtithorax* Pic, 1928. These characters are generally larger in female adults than male adults.

Light spectrum differences among sympatric fireflies

Table 1 and Figure 3 show λ_{max} of the light spectra of the flashes emitted from each species, ranging from 552 to 572 nm. *Curtos* and *Pyrocoelia* species had shorter λ_{max}, while those of *Luciola* species were longer. λ_{max} values were similar within the same genus, but different among different genera (Figure 3). The pair-wise comparison (Table 5) showed significantly different λ_{max} between species, except *Abscondita chinensis* (571.6±0.2 nm) versus *Luciola curtithorax* (570.5±0.5) and *Curtos sauteri* (553.5±0.4) versus larvae of *Pyrocoelia praetexta* (552.7±0.3).

We further compared the λ_{max} between interspecific females (Table 6). Most fireflies had significantly different λ_{max} between interspecific females, but those of *Aquatica ficta* (λ_{max} = 564.0 ± 0.5 nm) and *Abscondita cerata* (562.2 ± 0.4 nm) females were not significantly different. In contrast, several species showed no significantly different λ_{max} in their males (Table 7). Similar λ_{max} were found in males of *Luciola curtithorax*, *Abscondita chinensis,* and *Luciola kagiana*.

Light intensity between flashes and photic environment

Table 3 shows that fireflies start flashing or flying when the environmental light intensity decreased to 4.69–7.63 lux.

Molecular phylogeny inferred by DNA barcodes

The NJ (Figure 4) and ML trees (Figure 5) indicate that the genera *Abscondita*, *Curtos*, *Aquatica,* and *Luciola* belong to Luciolinae, while *Pyrocoelia* belongs to Lampyrinae.

Discussion

We identified five common species from Nankang and eight from Nanzhuang (Table 1). The evenings after sunny days with high humidity and cool temperature are the most suitable

for firefly activity (Table 3). Table 4 shows the differences in five major characters between females and males, all of which were generally larger in female adults than male adults. Along with their morphological differences, we also identified the light spectrum and light intensity of flashes related to the recognition of sympatric fireflies.

Light spectrum differences among sympatric fireflies

λ_{max} values were similar within the same genus, but different among genera (Figure 3). The pair-wise comparison (Table 5) also showed significantly different λ_{max} between species, except *Abscondita chinensis* versus *Luciola curtithorax* and *Curtos sauteri* versus larvae of *Pyrocoelia praetexta*. In addition, all but two species of fireflies had similar λ_{max} between intraspecific females and males. In *Abscondita cerata*, there was a slight difference in λ_{max} (p-value = 0.0109) between females (562.2±0.4 nm) and males (563.6±0.3). In *Luciola curtithorax*, there was an unexpected difference λ_{max} (p-value = 1.25E-12) between females (566.3±0.4 nm) and males (572.5±0.2). Further research is needed to explain such differences in gender. Nevertheless, most sympatric fireflies could distinguish between each other based on the light spectra of their flashes (Stanger-Hall & Lloyd 2015).

Most fireflies have significant different λ_{max} between interspecific females, but those of *Aquatica ficta* and *Abscondita cerata* females were not significantly different (Table 6). In contrast, similar λ_{max}'s were found in males of *Luciola curtithorax*, *Abscondita chinensis,* and *Luciola kagiana* (Table 7). However, different species had different flash patterns (data not shown). Thus, flash pattern is another key mechanism by which these sympatric male fireflies recognize females of their own species (Lewis & Cratsley 2008). Different light spectra are the major way in which most sympatric fireflies distinguish one another in the dark.

Light intensity of flashes reveals the putative communication distance

Fireflies seem to be very sensitive to environmental light intensity in the evening. Fireflies start flashing or flying when the environmental light intensity decreases to 4.69–7.63 lux (Table 3). Artificial light pollution is a major force influencing firefly proliferation, mating, and growth (Costin & Boulton 2016; Firebaugh & Haynes 2016; Haynes & Firebaugh 2019; Owens et al. 2018). Therefore, the light intensity of flashes emitted by fireflies could be an ecological indicator for evaluating light pollution. The average light intensity emitted by most females (from one segment of the light organ) is around half that of males (from two segments of the light organ) (Table 1). The average light intensity of all females except *Luciola kagiana* was 102–569 nW/cm², or ~0.7–4 lux. In contrast, the average light intensity of most males was 182–512 nW/cm², or ~1.3–3.5 lux. Furthermore, the maximum light intensity of males was 1.3–11-fold higher than that of females, which was 324–2048 nW/cm² or ~2.2–14 lux. Thus, an environmental light intensity suitable for firefly courtship could be established based on the above analysis.

Communication between female and male fireflies relies on the illumination of their light organ in the dark. The sensing distance between a female and male could be relative to their bioluminescent intensity. Thus, the maximum light intensity might represent the maximum sensing distance between females and males, assuming that the minimum sensing distance (r, meter) is around the same light intensity between females and males. One example is in the endemic *Abscondita cerata*, the males of which have a maximum light intensity of 2048 nW/cm² (14 lux) while that of the female is 187 nW/cm² (1.3 lux), which is $14 / (r^2) = 1.3$. Thus, the maximum sensing distance for this species is around 3.3 meters. With the same formula calculation, the average sensing distance is around 1.4 meters. In other words, the putative communication distance for *Abscondita cerata* could range from 1.4

to 3.3 meters, which is the sensing distance for a male searching for a female via flashing light.

Molecular phylogeny inferred from DNA barcodes

The cytochrome oxidase I (COI) barcode suggests a good resolution for species identification and a phylogeny that is consistent to those of previous morphological studies (Ballantyne et al. 2013; Ballantyne et al. 2015; Martin et al. 2017; Stanger-Hall et al. 2007) and mitochondrial phylogeny (Chen et al. 2019; Martin et al. 2017). The NJ (Figure 4) and ML trees (Figure 5) indicate that the genera *Abscondita*, *Curtos*, *Aquatica,* and *Luciola* belong to Luciolinae, while *Pyrocoelia* belongs to Lampyrinae.

Conclusion

This study establishes the light spectrum and intensity of nine sympatric fireflies, and can be referenced to ensure that light pollution in habitats does not become high enough to disrupt firefly mating. DNA barcoding revised the molecular phylogeny of the fireflies, which is consistent with those of previous morphological studies and could be applied for species identification.

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Competing interests: The authors declare that they have no conflict of interest.

Availability of data and materials: The mitogenomic sequences are in GenBank under accession numbers MT534191-MT534201.

Consent for publication: Not applicable.

Ethics approval consent to participate: All animal experiments in this study were performed in accordance with guidelines of the the Animal Ethics Committee of Academia Sinica.

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Table 1(on next page)

Light spectrum (λ_{max}) and intensity (nW/cm^2) of nine sympatric species from two habitats.

Table 1. Light spectrum (λ_{max}) and intensity (nW/cm²) of nine sympatric species from two habitats.

Species	Sex	Individuals (n)	λ_{max} (nm)	Light organ intensity (nW/cm²)	
				Mean	Maximum
A. Nankang, Taipei:					
Abscondita cerata	female	9	562.3 ± 0.4	164.6 ± 40.6	282.2
	male	14	563.2 ± 0.5	333.4 ± 91.3	1065
Aquatica ficta	female	-	-	-	-
	male	1	567	807.0	
Luciola kagiana	female	3	574.3 ± 0.3	NA	NA
	male	2	575.0 ± 0.0	5.4 ± 4.8	10.2
Luciola curtithorax	female	12	566.3 ± 0.4	157.9 ± 30.4	301.3
	male	26	572.5 ± 0.2	356.1 ± 48.0	814.1
Luciola filiformis	female	-	-	-	-
	male	12	567.3 ± 0.2	182.1 ± 31.2	323.8
B. Nanzhuang, Miaoli:					
Abscondita cerata	female	8	561.8 ± 0.8	102.0 ± 18.5	187
	male	14	564.1 ± 0.4	512.4 ± 198.3	2048
Abscondita chinensis	female	3	571.3 ± 0.3	245.7 ± 83.9	329.7
	male	2	572.0 ± 0.0	332.1	332.1
Aquatica ficta	female	5	564.0 ± 0.5	569.4 ± 101.1	850
	male	16	564.3 ± 0.2	508.1 ± 73.3	1102
Luciola kagiana	female	-	-	-	-
	male	1	572	NA	NA
Luciola curtithorax	female	-	-	-	-
	male	1	572	52.2	
Curtos sauteri	female	5	554.0 ± 0.3	187.7 ± 55.7	349.3
	male	3	552.7 ± 0.9	347.3 ± 95.9	536.7
Curtos costipennis	female	1	554	462	
	male	-	-	-	-
Pyrocoelia praetexta	larva*	3	552.7 ± 0.9	NA	NA

* light spectra were only successfully recorded from larvae.

Table 2(on next page)

DNA barcodes (COI) of studied fireflies.

Table 2. DNA barcodes (COI) of studied fireflies.

Species	Accession number (individual number)	Reference
Luciolinae:		
Abscondita cerata	MT534192 (6), MT534199 (3)	present study
Abscondita chinensis	MT534196 (1)	present study
Aquatica ficta	MT534197 (2)	present study
Curtos costipennis	AB608764	(Oba et al. 2011)
Curtos sauteri	MT534198 (1)	present study
Luciola curtithorax	MT534191(1), MT534193(1),MT534195(1)	present study
Luciola filiformis	MT534201 (1)	present study
Luciola kagiana	MT534200 (1)	present study
Lampyrinae:		
Pyrocoelia praetexta	MT534194 (1)	present study

Table 3(on next page)

The habitat temperature, relative humidity, and light intensity around twilight and when fireflies start flashing/flying.

Table 3. The habitat temperature, relative humidity, and light intensity around twilight and when fireflies start flashing/flying.

Date	Activity	Temp (°C)	RH (%)	Intensity (Lux)
A. Nankang, Taipei:				
4/20/2017	twilight	25.0±0.73	84.0±3.49	58.6±30.5
	start flashing/flying	22.6±0.14	95.8±0.47	4.69±2.37
4/29/2017	twilight	19.8±1.08	76.9±4.38	390.8±249.7
	start flashing/flying	17.1±0.83	87.3±1.67	5.40±3.09
5/1/2017	twilight	23.2±0.36	81.8±1.83	68.1±21.0
	start flashing/flying	21.2±0.45	92.0±2.41	7.63±6.91
5/18/2017	twilight	23.4±0.81	86.6±4.36	328.9±306.4
	start flashing/flying	22.3±0.17	92.9±0.94	7.61±4.76
B. Nanzhuang, Miaoli:				
4/28/2017	twilight	20.3±0.46	71.2±1.85	122.5±26.3
	start flashing/flying	17.9±0.87	86.6±4.04	5.94±3.47
5/7/2017	twilight	23.7±0.37	91.7±0.93	289.4±233.3
	start flashing/flying	22.7±0.40	94.8±1.23	6.12±3.61
5/8/2017	twilight	22.8±0.12	92.9±0.41	76.1±52.2
	start flashing/flying	22.0±0.29	95.1±0.73	6.35±3.69

Table 4(on next page)

Morphological measurements of eight adult fireflies.

Table 4. Morphological measurements of eight adult fireflies. TL: body length, PL: pronotum length, PW: pronotum width, EL: front wing length, and EW: front wing width.

Species	Specimen number	TL (mm)	PL (mm)	PW (mm)	EL (mm)	EW (mm)
Abscondita chinensis						
Female	3	10.49±0.26	2.03±0.1	3.1±0.2	8.12±0.49	3.88±0.10
Male	2	9.58±0.15	2.05±0.04	2.83±0.04	7.4±0.09	3.29±0.02
Total	5	10.12±0.5	2.03±0.08	2.99±0.21	7.83±0.52	3.64±0.30*
Abscondita cerata						
Female	28	10.01±0.7	2.02±0.18	3.14±0.28	7.77±0.56	4.05±0.32
Male	30	9.37±0.32	1.94±0.19	2.76±0.23	7.41±0.36	3.55±0.23
Total	68	9.69±0.63	1.98±0.19***	2.95±0.32	7.58±0.5	3.79±0.37
Aquatica ficta						
Female	5	9.5±1.04	2.03±0.11	3.03±0.34	7.46±0.85	3.67±0.41
Male	15	8.6±0.64	1.79±0.16	2.63±0.26	6.6±0.56	3.18±0.28
Total	20	8.83±0.85	1.85±0.18**	2.73±0.33	6.81±0.74	3.3±0.38
Luciola kagiana						
Female	1	10.52±0	2.08±0	3.06±0	8.65±0	3.85±0
Male	6	9.68±0.63	1.94±0.13	2.89±0.19	7.8±0.46	3.46±0.17
Total	7	9.8±0.65	1.96±0.13	2.91±0.19	7.92±0.52	3.52±0.21
Luciola curtithorax						
Female	2	6.67±0.11	1.28±0.19	2.08±0.03	5.02±0.02	2.61±0.1
Male	3	6.10±0.19	0.94±0.2	1.83±0.18	4.51±0.35	2.15±0.08
Total	5	6.33±0.32*	1.07±0.26	1.93±0.19	4.71±0.37	2.33±0.24
Luciola filiformis						
Female	0					
Male	1	5.93	1.26	1.53	4.63	2.07
Total	1	5.93	1.26	1.53	4.63	2.07
Curtos sauteri						
Female	4	6.43±0.58	1.28±0.2	1.88±0.28	5.07±0.57	2.47±0.19
Male	1	6.13	1.13	1.83	4.87	2.09
Total	5	6.37±0.53	1.25±0.19	1.87±0.26	5.03±0.51	2.39±0.23
Curtos costipennis						
Female	1	7.32	1.68	2.26	5.72	2.54
Male	0					

Total	1	7.32	1.68	2.26	5.72	2.54

* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001

Table 5(on next page)

Differences in pair-wise λ_{max} (p-value) between species.

The statistics were calculated using combined λ_{max} from females and males. Numbers in boldface are not significantly different.

Table 5. Differences in pair-wise λ_{max} (p-value) between species. The statistics were calculated using combined λ_{max} from females and males. Numbers in boldface are not significantly different.

	Ab. Cerata	*Ab. chinensis*	*Aq. ficta*	*C. sauteri*	*L. filiformis*	*L. curtithorax*	*L. kagiana*
Ab. chinensis	0.0000						
Aq. ficta	0.0012	0.0000					
C. sauteri	0.0000	0.0000	0.0000				
L. filiformis[a]	0.0000	0.0000	0.0000	0.0000			
L. curtithorax	0.0000	**0.0604**	0.0000	0.0000	0.0000		
L. kagiana	0.0000	0.0018	0.0000	0.0000	0.0000	0.0000	
P. praetexta[b]	0.0000	0.0000	0.0000	**0.1610**	0.0000	0.0000	0.0000

[a] only male; [b] only larva

Table 6(on next page)

Differences in pair-wise female λ_{max} (p-value) between species.

The statistics were calculated using λ_{max} of females. Numbers in boldface are not significantly different.

Table 6. Differences in pair-wise female λ_{max} (p-value) between species. The statistics were calculated using λ_{max} of females. Numbers in boldface are not significantly different.

	Ab. Cerata	*Ab. chinensis*	*Aq. ficta*	*C. sauteri*	*L. filiformis*	*L. curtithorax*
Ab. chinensis	0.0000					
Aq. ficta	**0.0742**	0.0000				
C. sauteri	0.0000	0.0000	0.0000			
L. filiformis[a]	NA	NA	NA	NA		
L. curtithorax	0.0000	0.0000	0.0095	0.0000	NA	
L. kagiana	0.0000	0.0000	0.0000	0.0000	NA	0.0000

[a] only male

Table 7 (on next page)

Differences in pair-wise male λ_{max} (p-value) between species.

The statistics were calculated using λ_{max} of males. Numbers in boldface are not significantly different.

Table 7. Differences in pair-wise male λ_{max} (p-value) between species. The statistics were calculated using λ_{max} of males. Numbers in boldface are not significantly different.

	Ab. cerata	*Ab. chinensis*	*Aq. ficta*	*C. sauteri*	*L. filiformis*	*L. curtithorax*
Ab. chinensis	0.0000					
Aq. ficta	**0.0672**	0.0000				
C. sauteri	0.0028	0.0021	0.0031			
L. filiformis[a]	0.0000	0.0000	0.0000	0.0022		
L. curtithorax	0.0000	**0.0626**	0.0000	0.0010	0.0000	
L. kagiana	0.0052	**0.1835**	0.0075	0.0001	0.0177	**0.2606**

[a] only male

Figure 1

Habitat locations.

A. Nankang, Taipei; B. Nanzhuang, Miaoli.

6/11/2020

Figure 1. Habitat locations. A. Nankang, Taipei; B. Nanzhuang, Miaoli.

Figure 2

Representative females and males of collected firefly species.

The standard scale bar is 1 mm, except for *Pyrocoelia praetexta* (5 mm scale bar).

Figure 2. Representative females and males of collected firefly species. The standard scale bar is 1 mm, except for *Pyrocoelia praetexta* (5 mm scale bar).

Figure 3

Light spectra (average λ_{max}) of the nine collected species.

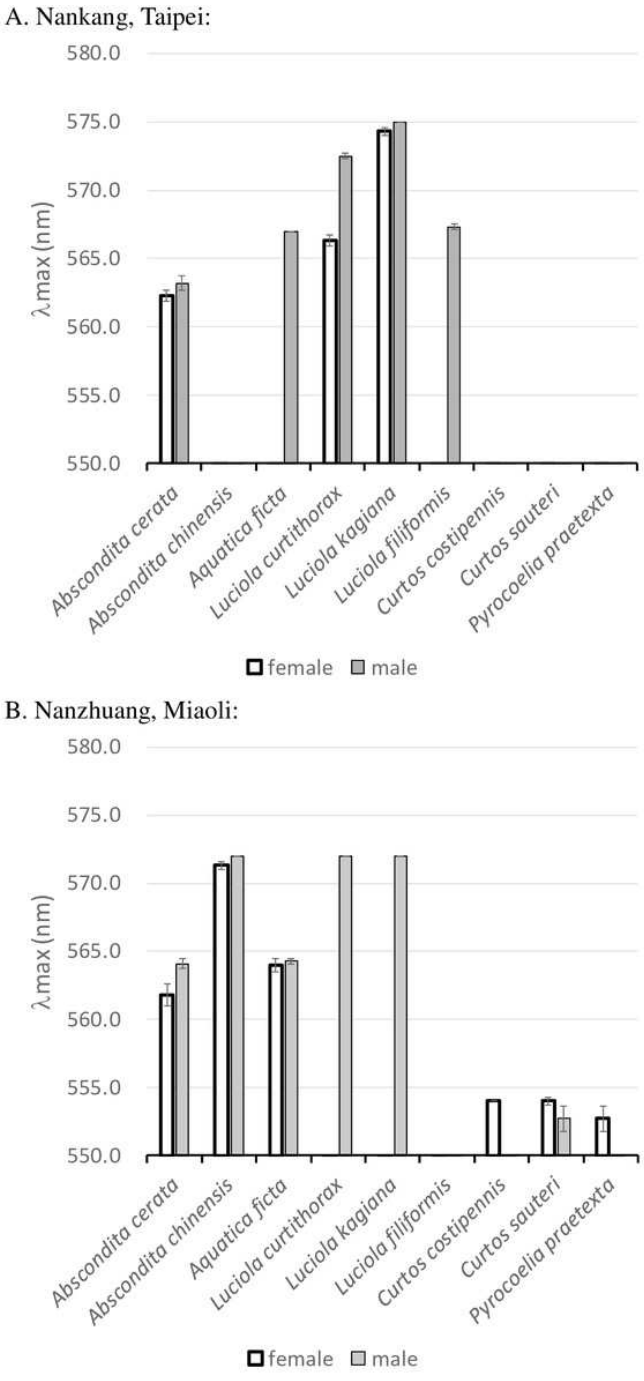

Figure 3. Light spectra (average λ_{max}) of the nine collected species.

Figure 4

Neighbor-Joining tree using the COI gene (520 bp) with bootstrap test results (500 replicates) at the nodes.

The optimal tree with the sum of branch length = 5.58552373 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) with number of base substitutions per site. The rate variation among sites was modeled with gamma distribution (shape parameter = 1.079137891). All positions with less than 95% site coverage were eliminated.

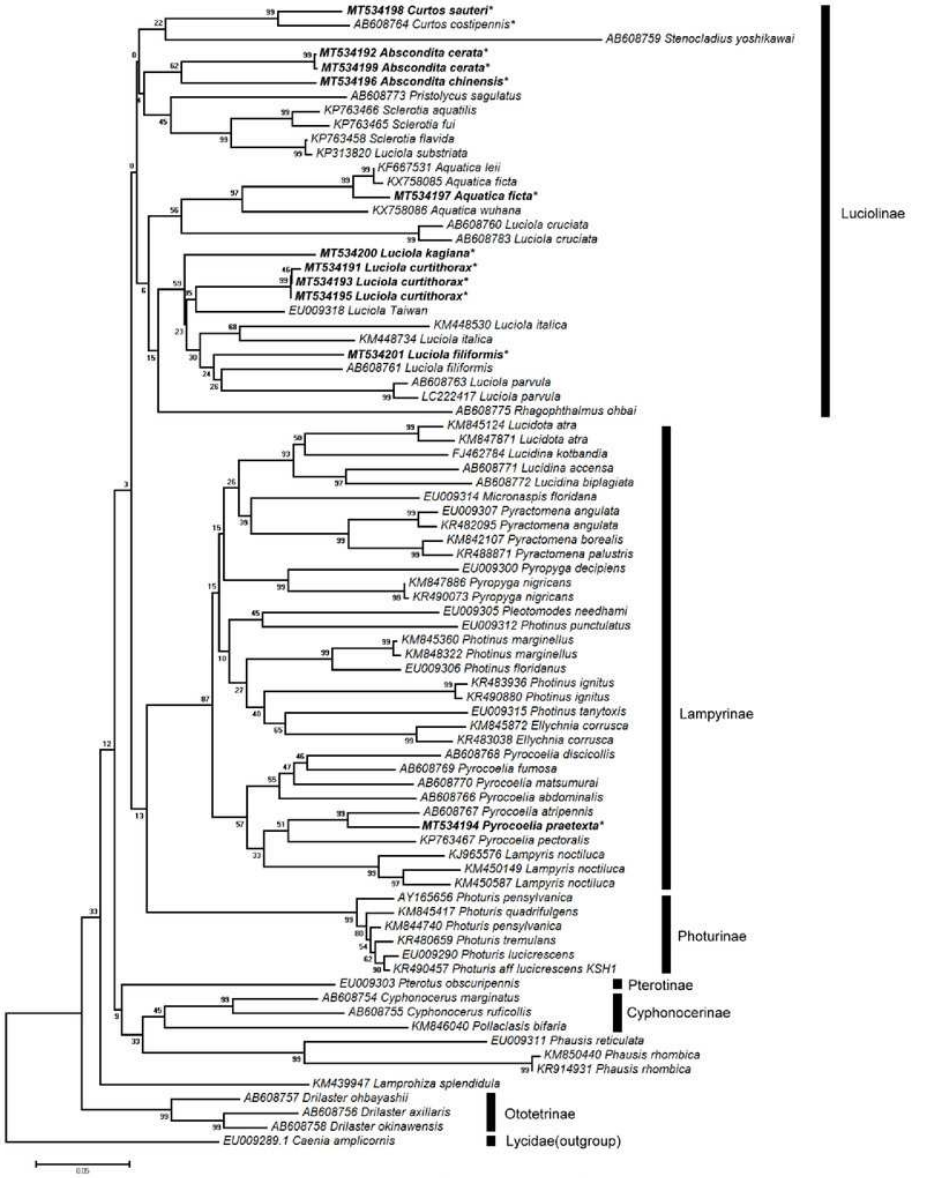

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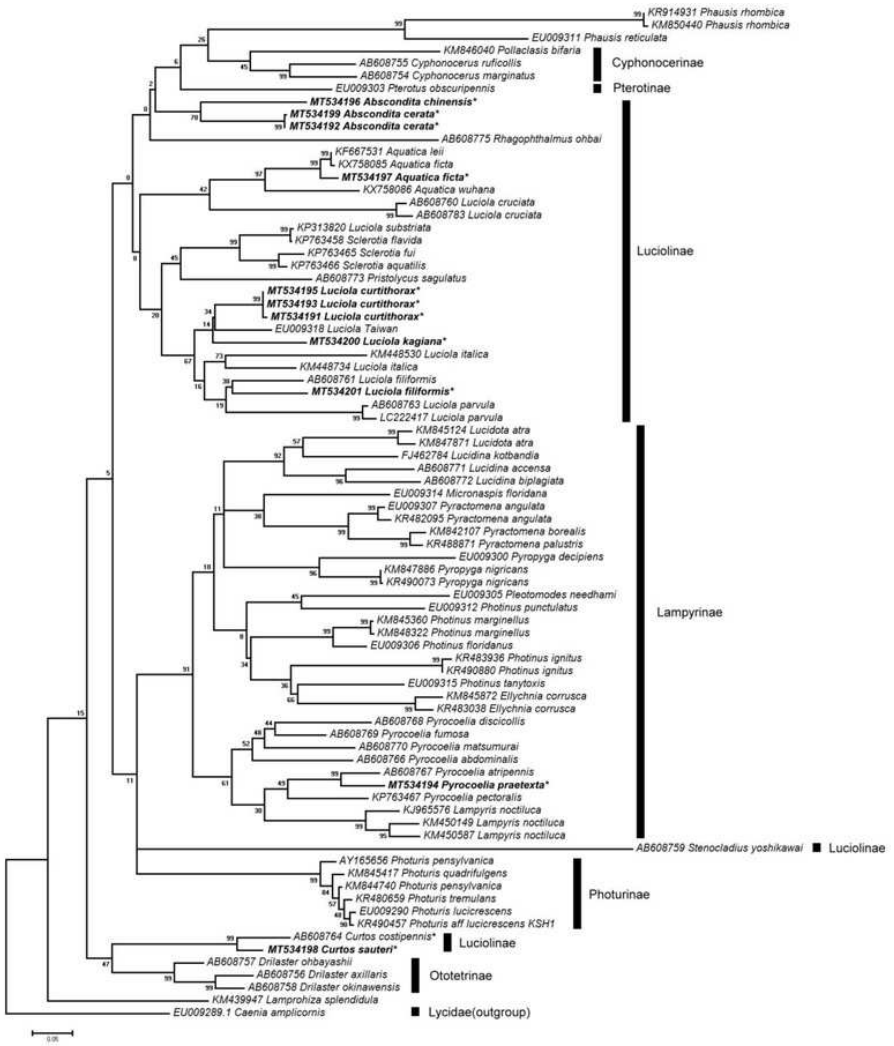
Figure 5

Maximum Likelihood tree using the COI gene (520 bp) with bootstrap test results (500 replicates) at the nodes.

The evolutionary history was inferred using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (-11653.0821) is shown. 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 the superior log likelihood value. A discrete gamma distribution was used to model differences in evolutionary rates across sites (4 categories (+G, parameter = 0.5737)). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 37.4868% sites). The tree is drawn to scale, with branch lengths measured based on the number of substitutions per site. All positions with less than 95% site coverage were eliminated.



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