

**SHORT REPORT**

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# Tests of conspecificity for allopatric vectors: *Simulium nodosum* and *Simulium shirakii* (Diptera: Simuliidae) in Asia

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## Abstract

**Background:** Allopatric populations present challenges for biologists working with vectors. We suggest that conspecificity can be concluded in these cases when data from four character sets—chromosomal, ecological, molecular, and morphological—express variation no greater between the allopatric populations than between corresponding sympatric populations. We use this approach to test the conspecificity of *Simulium nodosum* Puri on the mainland of Southeast Asia and *Simulium shirakii* Kono & Takahasi in Taiwan. The validity of these two putative species has long been disputed given that they are morphologically indistinguishable.

**Findings:** The mitochondria-encoded cytochrome c oxidase subunit I (COI), 12S rRNA, and 16S rRNA genes and the nuclear-encoded 28S rRNA gene support the conspecific status of *S. nodosum* from Myanmar, Thailand, and Vietnam and *S. shirakii* from Taiwan; 0 to 0.19 % genetic differences between the two taxa suggest intraspecific polymorphism. The banding patterns of the polytene chromosomes of the insular Taiwanese population of *S. shirakii* and mainland populations of *S. nodosum* are congruent. The overlapping ranges of habitat characteristics and hosts of *S. nodosum* and *S. shirakii* corroborate the chromosomal, molecular, and morphological data.

**Conclusions:** Four independent sources of evidence (chromosomes, DNA, ecology, and morphology) support the conspecificity of *S. nodosum* and *S. shirakii*. We, therefore, synonymize *S. shirakii* with *S. nodosum*. This study provides a guide for applying the procedure of testing conspecificity to other sets of allopatric vectors.

**Keywords:** *Simulium nobile* species group, Black flies, Phylogeny, Polytene chromosomes, Systematics, Multi-locus, Conspecificity

## Background

Simuliid vectors of human and animal pathogens typically are complexes of evolutionarily and ecologically distinct cryptic species, for which accurate identification is an essential first step in the epidemiological understanding and control of arthropod-borne diseases [1–4]. Compounding the challenge of recognizing cryptic species is the difficulty of evaluating the species status of allopatric populations. The problem is particularly acute when populations are widely disjunct on the mainland, between mainlands, or between an island and the mainland [5].

Assigning the same or different names to allopatric populations has consequences for understanding vector potential and developing control strategies [6].

A classic example of the challenge of evaluating species status for allopatric populations involves *Simulium nodosum* Puri, distributed from India across southern China, Myanmar, Thailand, and Vietnam [7], and the morphologically similar *Simulium shirakii* Kono & Takahasi from Taiwan, more than 130 km from the Chinese mainland. Although *S. shirakii* has been treated for 80 years as a separate species, its species status has long been questioned because of morphological similarity with *S. nodosum* [8]. *Simulium nodosum* is mammalophilic, attacking humans and bovids [9–11]. It is also a vector of the agents of filariasis to ruminants [10].

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Although allopatry confounds the evaluation of reproductive isolation, we suggest that allopatric populations, including putative species, can be considered conspecific when differences in their molecular sequences, chromosomal profiles, morphology, and ecology are within the range of variation for a geographically cohesive, panmictic set of populations. All four character sources require evaluation, given the existence of homosequential sibling species [12] and the failure of up to three of the character sets to discriminate reproductively isolated sympatric species [13].

We applied this test to two members of the *Simulium nobile* species group, *S. nodosum* and *S. shirakii*. The detailed morphological comparisons by Takaoka & Suzuki [8] between *S. nodosum* and *S. shirakii* originally suggested conspecificity and prompted the present study. We used a multi-locus phylogenetic analysis of one nuclear and three mitochondrial genes, a comparative analysis of the polytene chromosomes, and an evaluation of ecological data associated with our collections and in the literature [9, 14–16].

## Methods

No national permissions were required for this study, which did not involve endangered or protected species. No specific permissions were required to access the study sites; the collections were made on public lands.

Larvae were collected by hand into ethanol from five sites in Myanmar, Taiwan, Thailand, and Vietnam (Table 1). Additional samples of larvae from Taiwan were collected into 1:3 acetic ethanol for chromosomal comparison with published information [15]. Habitat characteristics at each collection site were recorded, including altitude, canopy cover, and stream depth, temperature, and width. Species identifications were performed using illustrated taxonomic keys [8, 14, 17–19].

The nucleotide sequences of the mitochondria-encoded COI, 12S rRNA, and 16S rRNA genes and the nuclear-encoded 28S rRNA gene were used. These genetic markers have been used to differentiate other simuliid species [20–23]. In addition to *S. nodosum* and *S. shirakii*, we included two nominal members of the *S. nobile* species group—*Simulium nobile* De Meijere from Gombak, Selangor, Malaysia (collected 23/07/14) and *Simulium timorensis* Takaoka, Hadi & Sigit from Kupang, Timor Island, Indonesia (collected 27/02/14)—for phylogenetic analysis.

Genomic DNA was extracted from each of five specimens per location, using the i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology Inc., Seongnam, South Korea). Amplifications of the mitochondria-encoded COI, 12S rRNA, and 16S rRNA, and nuclear-encoded 28S rRNA genes were undertaken in a final volume of 50  $\mu$ L containing 50–100 ng genomic DNA, 25  $\mu$ L

of ExPrime Taq Master Mix (GENETBIO Inc., Daejeon, South Korea), and 10 pmol of each forward and reverse primer. The primers used in this study were adopted from Folmer *et al.* [24] for COI, Kocher *et al.* [25] and Simon *et al.* [26] for 12S rRNA, Xiong & Kocher [20] for 16S rRNA, and Low *et al.* [27] for 28S rRNA.

Data on the nucleotide sequences of the COI, 12S rRNA, 16S rRNA, and 28S rRNA genes were deposited in the NCBI GenBank under accession numbers KP661441–KP661560. DNA sequences were analysed and edited using ChromasPro 1.5 (Technelysium Pty Ltd, Brisbane, Qld, Australia) and BioEdit 7.0.9.0. [28]. Statistical congruence was calculated using a partition homogeneity test implemented in PAUP 4.0b10 [29]. No significant differences were found among separate gene regions ( $P = 0.600$ ); hence, data were concatenated for further analyses (Fig. 1 and Table 2). Aligned sequences of single genes and the concatenated dataset were subjected to Bayesian inference (BI) analysis using MrBayes 3.1.2 [30], neighbour-joining (NJ) and maximum parsimony (MP) analyses using PAUP 4.0b10, and maximum likelihood (ML) analysis using Treefinder Version October 2008 [31]. *Simulium tani* Takaoka & Davies cytoform 'K' and *Simulium leparensis* Takaoka, Sofian-Azirun & Ya'cob were used as outgroups. To determine intra- and interspecific variation among species/population pairs, uncorrected ( $p$ ) pairwise genetic distances were calculated using PAUP 4.0b10.

We used the Feulgen technique and chromosomal slide-mounting procedures outlined by Adler *et al.* [32] to prepare the polytene chromosomes of 51 larvae from two sites (19 larvae from Guangfu, 32 larvae from Ruisui) in Taiwan (Table 1). Larval carcasses were deposited in the Clemson University Arthropod Collection. The chromosomes of all 51 larvae (25 females, 26 males) were compared band-for-band with the standard map for the subgenus *Simulium* [33, 34] and with the chromosomes of larvae analyzed by Tangkawanit *et al.* [15] from nine sites in Thailand.

## Findings

The phylogenetic tree for the concatenated dataset comprised two main clusters (Fig. 1). One cluster, consisting of *S. nobile* and *S. timorensis*, was supported with full posterior probability/bootstrap values (BI = 1.00, NJ = 100 %, MP = 100 %, ML = 100 %). The other cluster, consisting of *S. nodosum* and *S. shirakii* from different geographical regions, was supported with high to full posterior probability/bootstrap values (BI = 0.99, NJ = 100 %, MP = 100 %, ML = 100 %).

No phylogenetic tree from the concatenated dataset nor the single-locus analyses separated *S. nodosum* from *S. shirakii*. Both putative species were distributed randomly in the trees (Additional file 1: Figures S1–S4). The

**Table 1** Collection data for populations used in chromosomal and molecular analyses of *Simulium nodosum* and *S. shirakii*

Locality	Coordinates	Date	Taxon	Altitude	Width	Depth	Temperature	Canopy cover
San Village, Chiang-Tung, Myanmar	21°05'18.5"N 99°47'39.7"E	27/10/2013	<i>S. nodosum</i>	958 m	50 m	20 cm	22.7 °C	Open
Da Chais, Lam Dong, Vietnam	12°08'32.4"N 108°38'58.3"E	24/04/2014	<i>S. nodosum</i>	1439 m	3-6 m	NA	20.0 °C	Open
Doi Saket, Chiang Mai, Thailand	19°02'27.2"N 99°20'06.0"E	24/08/2014	<i>S. nodosum</i>	733 m	3.5 m	35 cm	22.7 °C	Open
Guangfu, Hualien, Taiwan	23°31'12.7"N 121°24'44.5"E	24/11/2008	<i>S. shirakii</i>	70 m	1 m	NA	22.0 °C	Shaded
Ruisui, Hualien, Taiwan	23°38'11.9"N 121°25'17.8"E	25/11/2008	<i>S. shirakii</i>	15 m	15 m	NA	22.0 °C	Open

NA not available

pairwise genetic distance analysis revealed intraspecific variation ranging from 0 to 0.23 % for *S. nodosum* from Vietnam, Myanmar, and Thailand; 0 to 0.08 % for *S. shirakii* from Taiwan; and 0 to 0.19 % for *S. nodosum/S. shirakii* (Table 2). The interspecific variation between *S. nodosum/S. shirakii* and *S. nobile*, and between *S. nodosum/S. shirakii* and *S. timorensis*, ranged from 5.10 to 5.45 % and 5.18 to 5.45 %, respectively. Thus, the level of polymorphism (0–0.19 %) between *S. nodosum* and *S. shirakii* was less than that between all pairs of mainland populations and within the Myanmar population (0.04–0.23 %) and far below that of *S. nobile* or *S. timorensis*.

Chromosomal banding patterns of all Taiwanese larvae were four fixed inversions (*IS-1*, *IIL-a*, *IIIL-b*, *IIIL-2*) removed from the *Simulium* subgeneric standard sequence, and matched the fixed banding sequence of all 247 Thai larvae studied by Tangkawanit *et al.* [14]. Taiwanese and Thai populations had the nucleolar organizer in the end of IIS, undifferentiated sex chromosomes, and only two autosomal polymorphisms each, one of which (*IIIL-1*) was shared. The frequency of *IIIL-1* was 0.99—nearly fixed—in Taiwan, and an average of 0.28 in Thai populations analyzed by Tangkawanit *et al.* [15]. The chromosome map of Tangkawanit *et al.* [15] for *IIIL*, however, shows the homozygous sequence for the *IIIL-1* inversion rather than the claimed standard sequence; in addition, the distal breakpoint is shown as extended by one band beyond the actual breakpoint. *IIL-1* was an infrequent inversion (average frequency = 0.04) in Thailand [15] and absent in Taiwan. *IL-1* was a new, but rare (0.01) inversion, with breakpoints before the last bands in each of sections 39 and 40, in one male larva in Taiwan. Ectopic pairing of centromeres formed a loose pseudochromocenter in populations in Thailand [15] but was present in only about 1 % of nuclei per larva in Taiwan.

Habitat characteristics for our collection sites of *S. nodosum* and *S. shirakii*, such as stream width, overlapped broadly. Altitude, however, was considerably

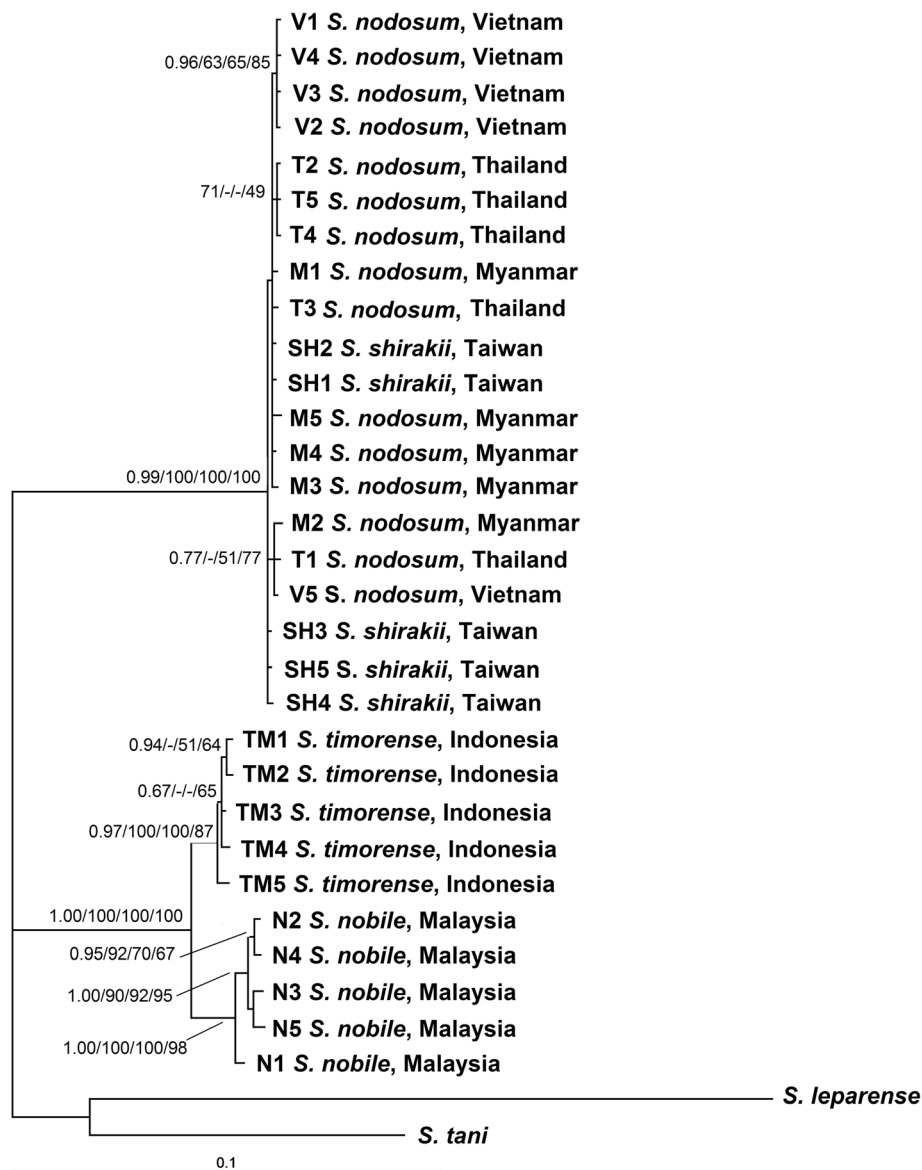
greater for our mainland populations (733–1439 m) than for our Taiwanese populations (15–70 m) (Table 1).

## Discussion

All four data sources—molecular, chromosomal, morphological, and ecological—independently support the conspecificity of *S. nodosum* and *S. shirakii* across a longitudinal range of 2365 km, providing a powerful consensus that *S. shirakii* and mainland populations in Myanmar, Thailand, and Vietnam are a single species. Our multi-locus analysis demonstrates that genetic differences within mainland populations of *S. nodosum* are greater than the differences between *S. nodosum* collectively and *S. shirakii*.

*Simulium nodosum* from Thailand and *S. shirakii* from Taiwan are identical in all details of their fixed chromosomal inversions and sex chromosomes, and share one of three autosomal inversions, the other two being rare. Inversion *IIIL-1*, which is found in about one-third of all homologues of Thai larvae and is nearly fixed in Taiwanese larvae, possibly expresses clinal variation, with lowest polymorphism in the Taiwan population, a common characteristic of insular and peripheral populations [32].

Habitat characteristics that typically differ between closely related species, especially stream size [35], are broadly overlapping among populations from Taiwan and the mainland, in agreement with records from the literature [14, 15]. Known hosts, which can differ between closely related species [36], are consistent (bovids and humans) across the distribution [9, 11, 14]. Altitude, however, which can be associated with genetic isolation [37], differs between our populations in Taiwan (15–70 m) and on the mainland (733–1439 m). The altitudinal distinction, however, narrows or disappears when we draw from published information [14], suggesting that some of the discrepancy is attributable to sampling artifact. Elevation for *S. nodosum* in Thailand, for instance, ranged from 168 to 800 m [15]. The available ecological data suggest that a



**Fig. 1** Bayesian inference phylogenetic tree of *Simulium* taxa based on concatenated COI, 12S rRNA, 16S rRNA, and 28S rRNA sequences. Posterior probability/bootstraps [Bayesian inference (BI)/neighbour-joining (NJ)/maximum parsimony (MP)/ maximum likelihood (ML)] values are shown on the branches. The scale bar represents 0.1 substitutions per nucleotide position

**Table 2** Ranges of intra- and interspecific genetic distances (uncorrected *p*, expressed as percentages) based on concatenated COI, 12S rRNA, 16S rRNA, and 28S rRNA sequences

	1	2	3	4	5	6
1. <i>S. nodosum</i> (Myanmar)	0.04–0.23					
2. <i>S. nodosum</i> (Vietnam)	0.04–0.23	0.00–0.15				
3. <i>S. nodosum</i> (Thailand)	0.04–0.23	0.04–0.19	0.00–0.19			
4. <i>S. shirakii</i> (Taiwan)	0.00–0.19	0.04–0.12	0.04–0.15	0.00–0.08		
5. <i>S. nobile</i>	5.18–5.41	5.22–5.41	5.14–5.45	5.10–5.37	0.08–0.35	
6. <i>S. timorensis</i>	5.22–5.41	5.29–5.41	5.26–5.45	5.18–5.37	1.00–1.27	0.04–0.23

broad range of habitat conditions is typical for *S. nodosum* and *S. shirakii*. Insular populations, in particular, are more likely to have a broader ecological niche, resulting in part from ecological release [38].

The molecular separation of *S. nodosum* and *S. shirakii* from *S. nobile* and *S. timorensis* is in concordance with their morphological characters. The simple claws of the females and the number and form of the pupal gill filaments (three inflated horn-like tubes) separate *S. nodosum*/*S. shirakii* from other members of the *S. nobile* species group [8].

## Conclusions

Given the conspecificity of *S. shirakii* with mainland populations, what taxonomic decision should be applied to populations across the entire range of *S. nodosum* and *S. shirakii*, from the type locality in India to Taiwan? Attempts by colleagues from India to collect *S. nodosum* from its type locality, approximately 2500 km from our nearest site of analysis (Thailand), did not produce *S. nodosum*. Lacking topotypical material for molecular and chromosomal analyses, we acknowledge two alternative possibilities: (1) recognition of two species—*S. nodosum*, represented by the type from India, and *S. shirakii*, represented by the material in our study, from Myanmar to Taiwan, or (2) recognition of a single species, *S. nodosum*, across the full range from India to Taiwan. We provisionally select the latter option on pragmatic grounds; the same name, when applied across the entire range, holds greater information content, emphasizing the morphological and ecological similarity of all populations. We, therefore, synonymize *S. shirakii* with *S. nodosum*.

## Additional file

**Additional file 1: Figure S1.** Bayesian inference phylogenetic tree of *Simulium* taxa based on COI sequences. Posterior probability/bootstrap [Bayesian inference (BI)/neighbour-joining (NJ)/maximum parsimony (MP)/ maximum likelihood (ML)] values are shown on the branches. The scale bar represents 0.1 substitutions per nucleotide position. **Figure S2.** Bayesian inference phylogenetic tree of *Simulium* taxa based on 12S rRNA sequences. Posterior probability/bootstrap [Bayesian inference (BI)/neighbour-joining (NJ)/maximum parsimony (MP)/ maximum likelihood (ML)] values are shown on the branches. The scale bar represents 0.1 substitutions per nucleotide position. **Figure S3.** Bayesian inference phylogenetic tree of *Simulium* taxa based on 16S rRNA sequences. Posterior probability/bootstrap [Bayesian inference (BI)/neighbour-joining (NJ)/maximum parsimony (MP)/ maximum likelihood (ML)] values are shown on the branches. The scale bar represents 0.1 substitutions per nucleotide position. **Figure S4.** Bayesian inference phylogenetic tree of *Simulium* taxa based on 28S rRNA sequences. Posterior probability/bootstrap [Bayesian inference (BI)/neighbour-joining (NJ)/maximum parsimony (MP)/ maximum likelihood (ML)] values are shown on the branches. The scale bar represents 0.1 substitutions per nucleotide position.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

VLL, PHA, MSA and HT contributed to the study design. MSA, WS, AS, YTH, UKH, XDP and HT contributed to specimen collection. VLL and PHA performed the experimental work. VLL and PHA analysed the data. VLL and PHA wrote the manuscript. All authors read and approved the final manuscript.

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