CSIRO PUBLISHING

Invertebrate Systematics, 2012, **26**, 445–450
http://dx.doi.org/10.1071/IS12029

DNA barcodes and morphology reveal a hybrid hawkmoth in Tahiti (Lepidoptera : Sphingidae)

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Abstract. Interspecific hybridisation is a rare but widespread phenomenon identified as a potential complicating factor for the identification of species through DNA barcoding. Hybrids can, however, also deceive morphology-based taxonomy, resulting in the description of invalid species based on hybrid specimens. As the result of an unexpected case of discordance between barcoding results and current morphology-based taxonomy, we discovered an example of such a hybrid 'species' in hawkmoths. By combining barcodes, morphology and a nuclear marker, we show that *Gnathothlibus collardi* Haxaire, 2002 is actually an F1 hybrid between two closely related species that co-occur on Tahiti. In accordance with the International Code of Zoological Nomenclature, the taxon *G. collardi* is thus invalid as a species. This study demonstrates the potential of DNA barcodes to detect overlooked hybrid taxa. With the growth of sequence libraries, we anticipate that more unsuspected hybrid species will be detected, particularly among those taxa that are very rare, such as those known from only the type specimen.

Additional keywords: 28S rDNA, COI, hybrid detection.

Received 17 April 2012, accepted 13 September 2012, published online 19 December 2012

Introduction

Natural interspecific hybridisation is a rare but widespread phenomenon in animal taxa (Mayr 1942; Seehausen 2004; Mallet 2008; Schwenk *et al*. 2008). Because it can lead to introgression between species, hybridisation can complicate the use of DNA-based approaches for species identification such as DNA barcoding (Vences *et al*. 2005; Whitworth *et al*. 2007; Schmidt and Sperling 2008) by causing discordance between the species-tree and the gene-tree in which species appear para- or polyphyletic (Funk and Omland 2003; Bachtrog *et al*. 2006; Zakharov *et al*. 2009). However, the complicating effects of hybridisation are not restricted to DNA-based identification methods; they can also strongly affect morphological identification (Thulin *et al*. 2006). Between closely related species that lack clear-cut diagnostic characters, hybrids can be confounded with intraspecific variation in one or other parental species. Undetected, they may blur the delineation of diagnostic characters between the two hybridising species. Alternatively, hybrids can be different enough from both parent species to gain description as a distinct species. Such cases have seldom been investigated in animals (but see Graves (1990) and Parham *et al*. (2001)), though they represent erroneous species accounts with a potential impact on taxonomy users. Resources may, for instance, be wasted on conservation of what are thought to be rare and endangered species, but which are actually hybrids (Allendorf *et al*. 2001). Interestingly, in cases where species might have been described erroneously on the basis of hybrid specimens, the discordance between morphological diagnostic characters and mtDNA offers a way of detecting a possible hybrid specimen, identifying its maternal parent species, and then triggering further study to confirm its status.

In this paper, we present a case study in which the investigation of a case of discordance between DNA barcodes and morphology revealed that *Gnathothlibus collardi* Haxaire, 2002, a rare hawkmoth species (Haxaire 2002), was described from specimens resulting from the hybridisation of two species on Tahiti. We present morphological and nuclear DNA evidence supporting this conclusion.

Materials and methods

Specimen sampling

In the course of the global DNA barcoding campaign for sphingid moths (or hawkmoths), specimens of *Philodila*

astyanor (Boisduval, [1875]), *Gnathothlibus collardi*, and *G. eras* (Boisduval, 1832) were sampled from Tahiti, the largest of the Society Islands in French Polynesia. Whereas the first two species are endemic to Tahiti, the third has a broad range extending through Melanesia to Sulawesi and the northern and eastern coasts of Australia. Samples analysed for this species include representatives from Tahiti, Bora Bora, Australia (Northern Territory, Queensland and Dauan Island) and Sulawesi. In total, 22 specimens were sampled for DNA barcoding and included in this study: 2 paratypes of *G. collardi*, 4 specimens of *P. astyanor*, and 16 specimens of *G. eras* (4 of these from Tahiti). Details of the specimens included in this study are given in Table 1; each record is given a unique specimen identifier (SampleID) and sequence identifier (ProcessID). Collection data, a photograph and ancillary information, such as the collection holding each specimen, are available in BOLD, the Barcode of Life Datasystems (Ratnasingham and Hebert 2007) in public projects.

DNA extraction, sequencing and data accessibility

The protocol for DNA extraction, amplification and sequencing followed the standard high-throughput procedure developed and used for Lepidoptera at the Canadian Centre for DNA Barcoding (see Vaglia *et al*. (2008) for details). This procedure includes a multistep iterative PCR strategy in which DNA samples that fail to amplify are 'hit-picked' and go through additional PCR amplification attempts targeting shorter amplicons (Decaëns and Rougerie 2008). After we realised that *Gnathothlibus collardi* specimens might be hybrids between *G. eras* and *Philodila astyanor*, we sought additional evidence by sequencing a nuclear marker. From the same DNA extracts used to generate the DNA barcode sequences, we amplified and sequenced the D2 expansion segment of the large ribosomal subunit (28S). This fragment was amplified using the primer pair D2B and D3Ar (Saux *et al*. 2004). The PCR cocktail was similar to that used for COI, but a different thermocycling regime was employed: 1 cycle of 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C, with a final step of 2 min at 72°C. Contigs were assembled using SeqScape ver. 2.1.1 (Applied Biosystems, Carlsbad, CA) and Sequencher 4.5 (Gene Code Corporation, Ann Arbor, MI), and were subsequently aligned using Bioedit ver. 7.0.5.3 (Hall 1999). The absence of indels in both the COI and 28S sequences made sequence alignment straightforward and unambiguous. Along with specimen data, sequence data for both genes are available on BOLD within public projects and in the assembled dataset SPHhyb01. All sequences are also available on GenBank with the accession numbers listed in Table 1.

Sequence analyses

The consistency of DNA barcodes as diagnostic molecular markers for the three taxa studied was tested through sequence similarity analysis, using the Management and Analysis component of BOLD (Ratnasingham and Hebert 2007) to compute K2P distances as a measure of intra- and interspecific variation. Neighbour-Joining (NJ) analysis, as implemented in BOLD, was used to construct the distance tree. Diagnostic characters in 28S rDNA sequences were searched for visually using Bioedit.

Table 1. List of the samples used for the genetic analysis

PT = paratype; Dep. = depository collection (RCJH: Research Collection of Jean Haxaire, Laplume, France; BIO: Biodiversity Institute of Ontario, Guelph, Canada; ANIC: Australian National Insect Collection, Canberra, Australia; RCYE: Research Collection of Yves Estradel, Le Vernet, France); F = female; M = male. SampleID codes are unique identifiers referring to individual records in the Barcode of Life Datasystems (BOLD, www.boldsystems.org); sequence lengths and GenBank accession numbers are given in separate columns for each gene

Taxon	Sex	Dep.	Country, region	Date coll.	SampleID	COI	28S
***Gnathothlibus collardi* PT**	M	RCJH	French Polynesia, Tahiti	08 Jun. 2002	BC-Hax1410	658 [JX438281]	602 [JX438295]
PT	M	RCJH	French Polynesia, Tahiti	01 Jul. 1986	BC-Hax1411	0	–
Gnathothlibus eras	M	RCJH	Australia, Dauan Island	26 Feb. 2004	BC-Hax4481	658 [HM384195]	–
	F	RCJH	French Polynesia, Tahiti	21 Jun. 1996	BC-Hax1416	658 [HM384048]	–
	M	RCJH	French Polynesia, Tahiti	21 Jun. 1996	BC-Hax1415	658 [JX438289]	602 [JX438300]
		BIO	Australia, Queensland	21 Feb. 2007	gvc6901–1 L	658 [JX438282]	–
		BIO	Australia, Queensland	21 Feb. 2007	gvc6904–1 L	658 [JX438293]	–
		BIO	Australia, Queensland	21 Feb. 2007	gvc6840–1 L	658 [JX438290]	–
	M	RCJH	French Polynesia, Tahiti	21 Jun. 1996	BC-Hax1417	658 [JX438278]	602 [JX438294]
	F	RCJH	French Polynesia, Tahiti	19 Jan. 2002	BC-Hax1418	608 [JX438284]	602 [JX438297]
	M	RCJH	Indonesia, Sulawesi	01 Jan. 2000	BC-Hax1401	609 [JX438287]	–
	M	RCJH	Indonesia, Sulawesi	04 Dec. 2000	BC-Hax1402	609 [JX438285]	–
	F	RCJH	Indonesia, Sulawesi	01 Nov. 2001	BC-Hax1403	607 [JX438291]	–
		ANIC	Australia, Queensland	28 Feb. 1993	ANIC Gen No. 003230	658 [JX438280]	–
		ANIC	Australia, Queensland	18 Apr. 1994	ANIC Gen No. 003251	658 [JX438277]	–
		ANIC	Australia, Northern Territory	19 May 2001	ANIC Gen No. 003252	658 [JX438292]	–
		ANIC	Australia, Northern Territory	25 May 2001	ANIC Gen No. 003253	658 [JX438279]	–
	F	RCYE	French Polynesia, Bora Bora	04 Aug. 1995	BC-EST0585	658 [GU703969]	–
Philodila astyanor	M	RCJH	French Polynesia, Tahiti	21 Jun. 1996	BC-Hax1342	658 [JX438286]	–
	M	RCJH	French Polynesia, Tahiti	18 May 2002	BC-Hax1343	631 [JX438283]	602 [JX438296]
	M	RCJH	French Polynesia, Tahiti	18 May 2002	BC-Hax1344	656 [JX438288]	602 [JX438299]
	M	RCJH	French Polynesia, Tahiti	26 Mar. 2001	BC-Hax1345	658 [JN678365]	583 [JX438298]

Morphological examination

We examined several dozen specimens of *Gnathothlibus eras*, either directly in collections or as photographs, three male specimens of *G. collardi* including the holotype and two paratypes (to our knowledge, there is only one other specimen known for that species), and 37 specimens of *Philodila astyanor*, including a photograph of the holotype preserved at the Carnegie Museum of Natural History (CMNH, Pittsburgh, USA). The male genitalia of the three taxa were prepared using standard techniques and also compared.

Results

DNA barcoding

The COI region used as the standard DNA barcode in animals was recovered in all samples except for one of the paratypes of *Gnathothlibus collardi*, the age of which (>20 years old) probably prevented amplification of the target sequence despite several attempts to amplify shorter fragments (see Material and methods). In total, 21 barcodes were recovered representing four specimens of *Philodila astyanor*, 16 of *G. eras* and one of *G. collardi*. Sequence similarity analysis using K2P distances revealed a clear discrimination between the first two species, each of which formed a cohesive genetic unit as illustrated in the NJ tree (Fig. 1*B*). The minimum sequence divergence observed between the two species is 4.99%; all four specimens of *P. astyanor* possessed an identical DNA barcode, while *G. eras* specimens show some intraspecific variation (maximum and mean intraspecific distance of 0.83% and 0.35% (s.e. = 0.023%, $n = 16$) respectively). The presence of a clear barcode divergence was not surprising given the obvious morphological differences between these two species (Fig. 1*A*). However, our analysis unexpectedly revealed that the paratype of *G. collardi* possessed a barcode sequence identical to that of *P. astyanor* (Fig. 1*B*) despite the morphological dissimilarity between the two taxa (*G. collardi* resembles *G. eras* at first glance (Fig. 1*A*), and its male genitalia are also more similar to those of *G. eras* (Figs 2*B*, *C*, compared with 2*A*)). Cross-contamination between the two species was ruled out by reprocessing a second tissue sample from the same *G. collardi* specimen, which yielded identical results.

Test of the hybrid hypothesis

The barcode congruence between the specimen of *G. collardi* and *P. astyanor* could be explained if *G. collardi* specimens are F1

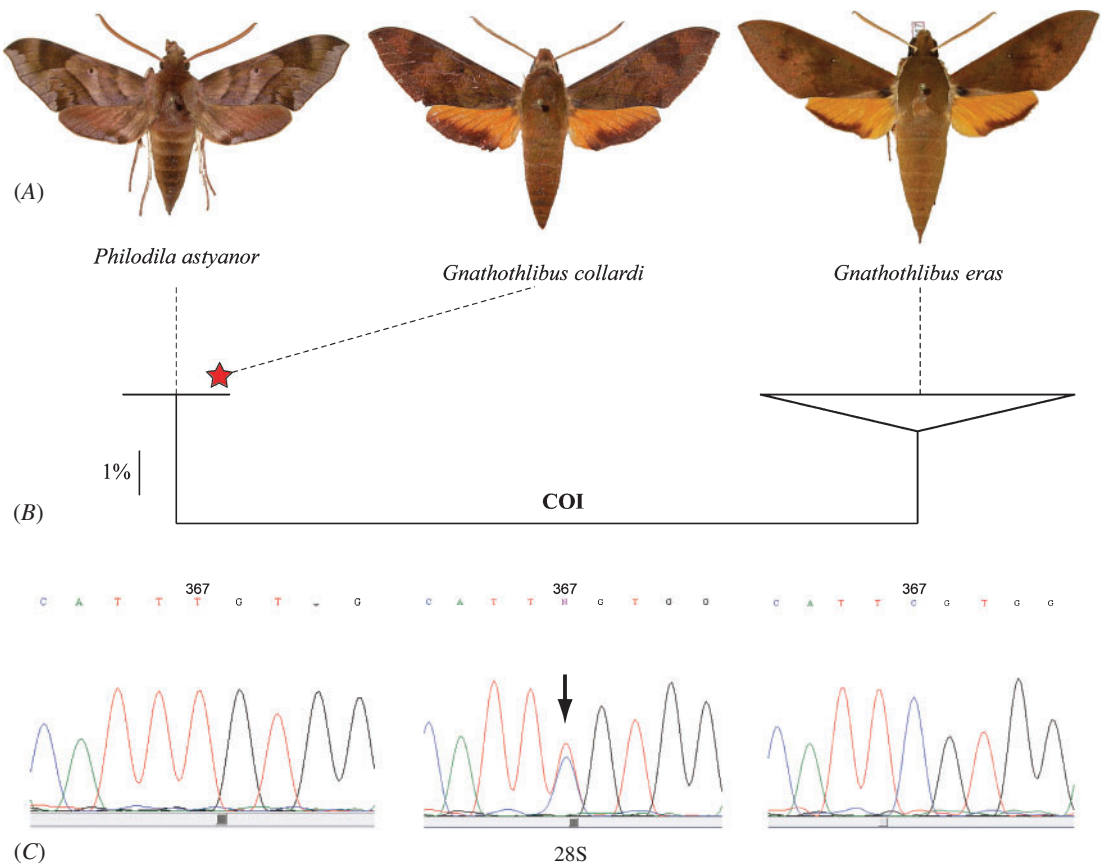

Fig. 1. (*A*) Males of the three studied taxa, dorsal view; all three collected in Tahiti, French Polynesia. (*B*) Neighbour-Joining tree representing DNA barcode sequence similarities among the 21 specimens of the three taxa analysed; the depth of the triangle represents the maximum intraspecific variation within *Gnathothlibus eras*. (*C*) Electropherograms for 28S rDNA sequences showing the diagnostic locus for *Philodila astyanor* and *G. eras* at Position 367 of the fragment analysed, and its heterozygous condition in *G. collardi* (arrow).

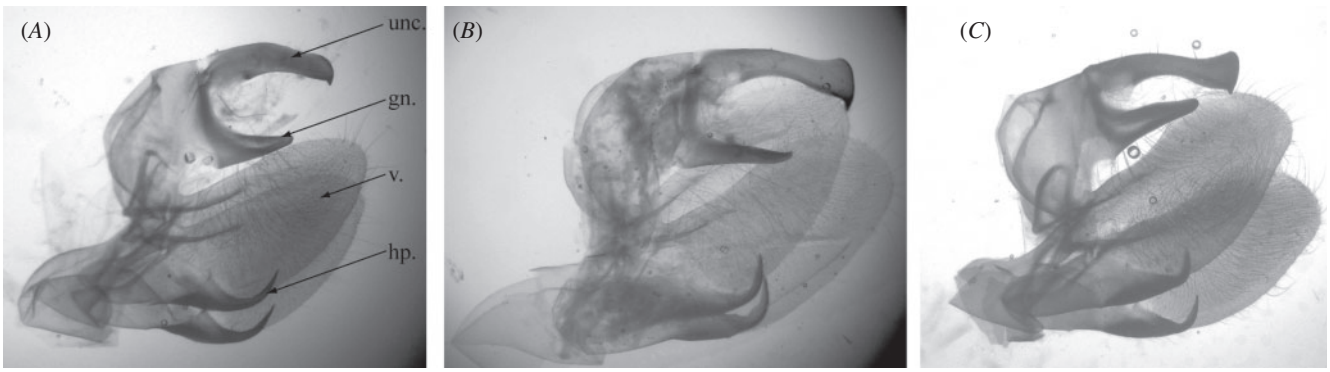

Fig. 2. Male genitalia, lateral view, of (*A*) *Philodila astyanor*, (*B*) *G. collardi*, and (*C*) *G. eras*. Arrows in (*A*) point at the morphological structures listed in Table 2: unc., uncus; gn., gnathos; v, valve; hp, harpe.

hybrids between female *P. astyanor* and male *G. eras*. This hybrid hypothesis would explain the morphological distinctiveness of *G. collardi* as well as its shared DNA barcode sequence through maternal inheritance of the mitochondrial genome. Examination of the D2 region of the 28S rDNA gene – a nuclear gene inherited from both parents – from three specimens each of the hypothetical parent species, revealed no variation except for a single diagnostic substitution distinguishing *P. astyanor* and *G. eras*. The two species showed a consistent difference at Position 367 of the amplified fragment of 28S (Fig. 1*C*), with a thymine in *P. astyanor* versus a cytosine in *G. eras*. In *G. collardi*, this position is heterozygous, as electropherograms show a cytosine + thymine double peak (Fig. 1*C*), supporting the hybrid hypothesis. A more thorough morphological reexamination of specimens of the three species then revealed that *G. collardi* displays a mosaic of characters found in its parent species (Table 2; Figs 1*A*, 2*A–C*). Because the species name, *Gnathothlibus collardi*, is based on a type specimen of hybrid origin, it is invalid (ICZN 1999, Article 23.8: '*A species-group name established for an animal later found to be a hybrid must not be used as the valid name for either of the parental species*').

Discussion

DNA barcodes revealed an unexpected case of a species invalidly described from hybrid specimens. The initial discordance between morphological identifications and the cohesiveness of DNA barcode clusters provoked deeper investigation of the situation with both a nuclear marker and morphology. These analyses showed that natural hybrid specimens between *Philodila astyanor* and *G. eras* in Tahiti had deceived a taxonomist into describing an invalid species. This represents the second known example in the Sphingidae as a similar case was recently reported

Table 2. Details of the mosaic of morphological characters (habitus and male genitalia) found in *Gnathothlibus collardi* and in the two parental species *Philodila astyanor* and *G. eras*

All characters of habitus and male genitalia are visible in Fig. 1*A* and in Fig. 2, respectively. Cells are coloured to illustrate similarities between the hybrid and its parental species

Characters	*Philodila astyanor*	*Gnathothlibus collardi*	*Gnathothlibus eras*
Habitus			
Hindwing general coloration	Brown	Orange	Orange
Forewing pattern	Posterior margin at base of wing lacking a dark spot	Posterior margin of forewing base with a dark spot	Posterior margin of forewing base with a dark spot
	Wing strongly marked by several darker patches: one postmedial (between vein M2 and the costal), one apical, and one at the tornus	Wing weakly marked by several darker patches: one postmedial (between vein M2 and the costa), one apical, and one at the tornus	No defined darker patches
	Ante- and postmedial bands strongly marked	Ante- and postmedial bands weakly marked but visible	Ante- and postmedial bands absent
Forewing shape	Outer margin produced strongly convex at end of vein M2	Outer margin produced weakly convex at end of vein M2	Outer margin evenly curved
Male genitalia			
Valve (lateral view)	Harpe inner edge evenly curved upward	Harpe inner edge curved upward but with a slight angle	Harpe inner edge angled upward at nearly 90°
	Apex narrowly rounded	Apex broadly rounded	Apex rounded-rectangular
Uncus (lateral view)	Dorsal edge flat, apex strongly and evenly convex	Dorsal edge slightly produced upwards, apex weakly convex	Dorsal edge strongly produced upwards, apex truncate
Gnathos (lateral view)	Deeper than long	Longer than deep	Longer than deep

by Hundsdoerfer *et al*. (2011), who inferred a hybrid origin for *Hyles sammuti* Eitschberger, Danner & Surholt 1998 from the discordance between morphology and mtDNA sequence data. However, they suggested retaining the name *sammuti* for convenience while awaiting confirmation from nuclear DNA sequences.

Although it is difficult to estimate how frequently taxonomists have erroneously described species from hybrids, we note that DNA barcode libraries assembled in collaboration with expert taxonomists can efficiently reveal cases where the interspecific hybrids differ phenotypically from their parent species. Rare species, such as those known only from the type specimen, may well involve a higher incidence of hybrid taxa. This status could explain why some species elude modern re-collection and are thought to be extinct. For instance, the African hawkmoth *Hippotion chloris* Rothschild & Jordan, 1907 is known only from the holotype, and Carcasson (1968) suggested that it was a hybrid between *Hippotion celerio* (Linnaeus, 1758) and *Basiothia medea* (Fabricius, 1781) because of its intermediate appearance between these taxa. Barcode records reveal that the two hypothetical parent species are more than 4% divergent (Wilson *et al*. 2011), with nearest neighbours respectively at 2.3% (*Hippotion aurora* Rothschild & Jordan, 1903) and 3.3% (*Basiothia laticornis* (Butler, 1879)). As a result, the recovery of even a short fragment of the barcode region from the holotype of *H. chloris*, as has already been achieved successfully for archival material in Lepidoptera (Hausmann *et al*. 2009*a*, 2009*b*), would allow for quick support or refutation of the hybrid hypothesis. In hawkmoths, rarity and intermediate phenotypes suggest that a similar hybrid status may also be hypothesised for *Xylophanes clarki* Ramsden, 1921, a possible hybrid between *X. tersa* (Linnaeus, 1771) and *X. pluto* (Fabricius, 1777), and *Hyles churkini* Saldaitis & Ivinskis, 2006, a possible hybrid between *H. zygophylli* (Ochsenheimer, 1808) and *H. euphorbiae* (Linnaeus, 1758) or *H. costata* (von Nordmann, 1851).

A high incidence of hybridisation has been reported in some groups of Lepidoptera, ranging from 6–15% within the genus *Papilio* (Sperling 1990) to as high as 29% within the tribe Heliconiina (Mallet *et al*. 2007). These reports suggest that F1 hybrids may account for several species, especially in groups like butterflies, hawkmoths and silkmoths where a significant fraction of species have been described from one or a few specimens. Taxonomic 'exaggeration' (Pillon and Chase 2007) caused by hybrids is certainly known from other groups that have seen intensive taxonomic study, including hummingbirds (Graves 1990) and turtles (Stuart and Parham 2007). Given the significant proportion of species described upon a single specimen, one in six newly described species of invertebrates during the past decade, and one in four for vertebrates (Lim *et al*. 2012), it is worth testing to determine how many were based on hybrid specimens.

DNA barcode reference libraries assembled with the involvement of expert taxonomists can help to reveal the impact of hybridisation on taxonomy, limiting the consequences of 'bad taxonomy' (Bortolus 2008). With possibly increasing rates of hybridisation and introgression as the result of species-range alteration by human activities (through modifications of habitats, translocations of organisms, climatic changes, etc.), interspecific hybrids may cause increasing taxonomic confusion. Importantly, these hybrid specimens or populations may have important implications for conservation (Brownlow 1996; Allendorf *et al*. 2001; vonHoldt *et al*. 2011) and their early detection can be critical to preventing local or even global extinction of parent species ('speciation reversal', see e.g. Vonlanthen *et al*. (2012)). In this respect, DNA barcode libraries may act as sentinels for the early detection of hybridisation.

Acknowledgements

We are particularly grateful to staff at the Canadian Centre for DNA Barcoding (University of Guelph, Canada) for their help in processing the specimens examined in this study. We thank Dr John Rawlins (Carnegie Museum of Natural History), who hosted IJK during his visits to the CMNH in 1992 and 1998, as well as Dr John Lasalle (Australian National Insect Collection, Canberra), Steeve Collard, Dominique Bernaud, Yves Estradel and Georges Orhant for contributing material in their collections or for sharing specimens they collected. Special credit is due to Steeve Collard who suggested the hybrid status when he reported the capture of the first specimen of *Gnathothlibus collardi* to JH; he abandoned this notion after collecting the third specimen on the basis that hybrids are exceptional in nature. Funding was provided by the government of Canada through Genome Canada and the Ontario Genomics Institute in support of the International Barcode of Life project, and by NSERC.

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