DNA barcoding identifies the first North American records of the Eurasian moth, *Eupithecia pusillata* (Lepidoptera: Geometridae)

JEREMY R. deWAARD[1,2], LELAND M. HUMBLE[1,3] and B. CHRISTIAN SCHMIDT[4]

ABSTRACT

The first North American records of the juniper pug moth, *Eupithecia pusillata* (Denis & Schiffermüller, 1775) (Lepidoptera: Geometridae), brought to our attention using DNA barcoding, are presented. Documentation and collection localities suggest it was introduced, established, and likely has persisted, at least in the Greater Vancouver area of British Columbia since the mid-1970s. We discuss the integration of DNA barcoding into routine biosurveillance and forest insect surveys to prevent such delay in recognition of non-indigenous species—in this case, 34 years.

Key Words: *Eupithecia pusillata*, *Eupithecia interruptofasciata, Eupithecia niphadophilata,* juniper pug moth, *Juniperus*, non-indigenous species, invasive species, DNA barcoding

INTRODUCTION

DNA barcoding of biological specimens has demonstrated repeatedly its utility as a molecular diagnostic technique that merits integration into biosurveillance programs. In contrast to other molecular tools commonly employed for species identification of intercepted organisms, DNA barcoding is a generic and standardized approach that meets international standards of data quality and transparency (Floyd et al. 2010). Several studies have demonstrated the efficacy of this technique for detecting non-indigenous species and determining native provenance, for example in leeches (Siddall and Budinoff 2005), agromyzid leafminers (Scheffer et al. 2006), tephritid fruit flies (Armstrong and Ball 2005; Barr 2009), siricid wasps (Wilson and Schiff 2010), true bugs (Nadel et al. 2010), and numerous taxa of moths (Ball and Armstrong 2006; Simonsen et al. 2008; Humble et al. 2009; deWaard et al. 2009; Gilligan and Epstein 2009; Armstrong 2010). Here we report the first North American records of the juniper pug moth, *Eupithecia pusillata* (Denis & Schiffermüller, 1775) revealed by DNA barcoding.

MATERIALS AND METHODS

While compiling a DNA barcode library for the Geometridae of British Columbia (deWaard et al., submitted), the cytochrome *c* oxidase subunit I (COI) sequences de-

[1] University of British Columbia, Department of Forest Sciences, Forestry Sciences Centre, Vancouver, BC, Canada V6T 1Z4
[2] Royal British Columbia Museum, Entomology, 675 Belleville Street, Victoria, BC, Canada V8W 9W2 (email: jdewaard@interchange.ubc.ca)
[3] Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, 506 West Burnside Road, Victoria, BC, Canada V8Z 1M5 (email: Leland.Humble@nrcan-rncan.gc.ca)
[4] Canadian Food Inspection Agency, Canadian National Collection of Insects, Arachnids and Nematodes, K.W. Neatby Building, 960 Carling Avenue, Ottawa, ON, Canada K1A 0C6 (email: Chris.Schmidt@inspection.gc.ca)

rived from two *Eupithecia* specimens were found to be divergent from known native *Eupthecia.* The two sequences were compared to a reference barcode database of Lepidoptera barcodes using the identification engine (BOLD-ID) of the Barcode of Life Data Systems (BOLD) (Ratnasingham and Hebert 2007), and tentatively identified as *Eupithecia pusillata*, a Eurasian species not known to occur in North America. The reference barcode database for Geometridae used by BOLD-ID is continually validated by specialists to ensure accurate identifications, and is particularly well parameterized due to a global campaign to barcode the nearly 23,000 species of the family (see http://www.lepbarcoding.org/geometridae/index.php). The nine sequences with identical and near-identical matches from Europe were obtained from Axel Hausmann (Zoological State Collection, Munich, Germany) and Marko Mutanen (University of Oulu, Oulu, Finland) and combined with related North American specimens (*sensu* Bolte 1990). A neighbour-joining tree was constructed on BOLD using the Kimura-2-parameter distance method (Fig. 1).

To pursue confirmation of the identity of the specimens, the two putative *E. pusillata* specimens obtained from the RBCM (Royal British Columbia Museum, Victoria, BC) and PFCA (Arthropod reference collection, Pacific Forestry Centre (PFC), Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Victoria, BC), were dissected to examine the genitalia following the methods given by Lafontaine (2004). Images of genitalia were taken using a Leica M205C microscope equipped with a Leica DFC490 camera kit and Leica LAS Montage system that assembles multiple images in successive planes of focus into a single image with a large depth of field. The specimens were verified by comparison of the structure of genitalia with specimens held in the CNC (Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa, ON), and figures of *E. pusillata* in Skou (1986) and Mironov (2003). Related species in the *E. niphadophilata* Dyar, 1904 group (Bolte 1990) were ruled out by genitalic comparison to specimens in the CNC, as were other North American species.

Historical data associated with the specimens were compiled from specimen labels and Forest Insect and Disease Survey (FIDS) records (Van Sickle et al. 2001). The single specimen from PFCA, collected by FIDS, is uniquely identified by a registration number (e.g. 76-9-0019-01) that links the specimen to a FIDS sampling form, completed at the time of sample collection, as well as a rearing record documenting the status of laboratory rearings. These records are held on file at PFC.

RESULTS

Specimens examined: 1♂ – *label data* (handwritten information in italics, individual lines separated by comma, multiple labels separated by '|'):

No. *76-9-0019-01*, Date *19 vii*, F.I.[D.] S.*1976* | *c. juniper*, *Port*, *Coquitlam BC* | Ac. No. PFC, 2007-0271.

The specimen was initially identified as *Eupithecia unicolor* (Hulst). The FIDS records document that this specimen was one of two adults reared from five larvae and five pupae (10 individuals in total) collected by the B.C. Forest Service on Mt. Burke, Port Coquitlam (UTM 10 53 546 [49.3, -122.7], Elevation 900 ft), on 15 May 1976. The host recorded was common juniper (*Juniperus communis* L.); Remarks & Symptoms state "Attacking several ornamentals with moderate damage". The date recorded on the specimen label is the date of adult eclosion. While the Rearing Record indicates a second adult eclosed on 8.vii.76 and was subsequently spread, the specimen could not be found in the PFCA reference collection.

1♀ – *label data*:

BC, N. Vancouver, 5 AUG 1986, C.S. Guppy | ROYAL BRITISH, COLUMBIA MUSEUM, ENT991-12573 |.

This specimen was identified as

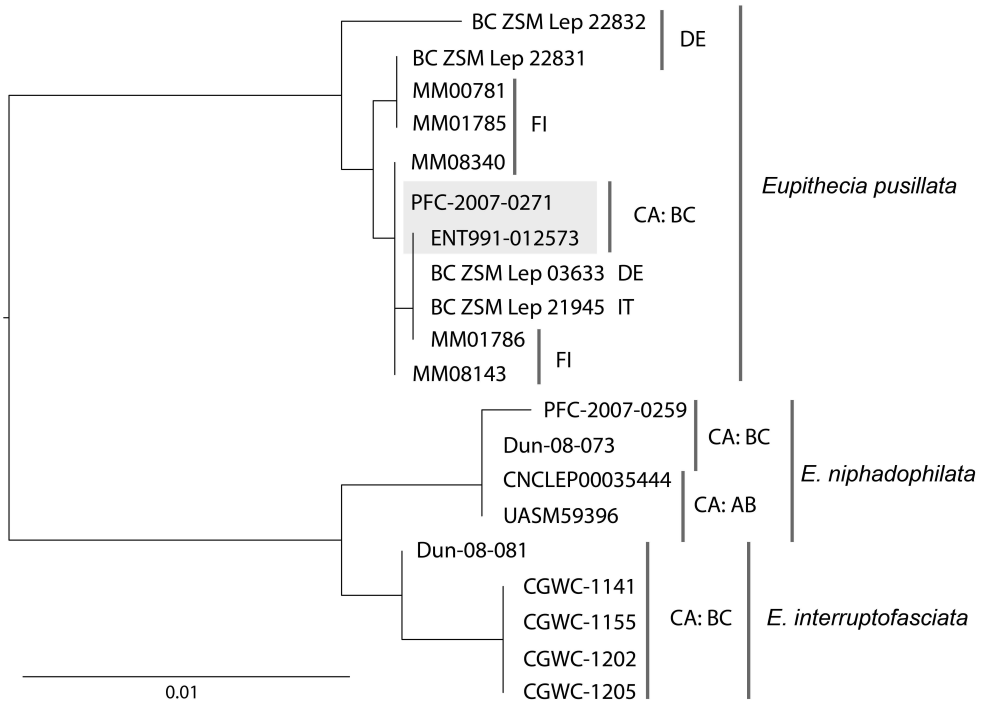

Figure 1. Neighbour-joining tree of *Eupithecia pusillata* and two closely related species, *E. niphadophilata* and *E. interruptofasciata*. Tree was reconstructed with the barcode fragment of the cytochrome oxidase I (COI) gene. Sequences shaded in grey are from two individuals collected in Vancouver, Canada. Abbreviations: DE – Germany, FI – Finland, IT – Italy, CA – Canada, BC – British Columbia, AB – Alberta.

Eupithecia sp. in the collection before tentative assignment to *Eupithecia intricata taylorata* Swett by JRD.

Diagnosis: *Eupithecia pusillata* is most similar to *E. niphadophilata* and particularly *E. interruptofasciata*, but a number of *Eupithecia* species are superficially very similar and identification should be based on examination of genitalia. Compared to *E. interruptofasciata*, which is structurally most similar, the male 8th sternite apical prongs are narrower, more blunt and the apical cleft is shallower; the base of the sternite is also narrower overall with a shallower medial invagination. The basal half of the male vesica is armed with one spine, not two as in *E. interruptofasciata.* In the female genitalia, the large spines on the left side of the ductus bursae do not extend beyond the mid-point of the ductus, but extend beyond the midpoint in both *E. interruptofasciata* and *E. niphadophilata*.

Description: A small moth with a wingspan of 16–22 mm (Mironov 2003) (Figs. 2a, 2e). Forewing narrow, mostly shades of light brown with black transverse lines and oblong discal spot. Hindwing pale greybrown with weakly marked transverse lines and variable discal spot. Abdomen pale grayish brown with narrow black lateral stripes. Male genitalia (Fig. 2d) composed of broad valva with small ventral process, heavily sclerotized sacculus, vesica with three horn-like cornuti, simple aedeagus (Fig. 2c) and elongated 8th sternite with two narrow apical processes (Fig. 2b). Female genitalia composed of elongate and sclerotized bursa copulatrix (Fig. 2h) with small spines at base and larger spines at margin. Ovipositor is simple with long setae (Fig. 2f). Terminal segment of pupal case is stout with prominent lateral lobes and cremaster bearing four pairs of hook-like setae (Figs. 2h, 2i).

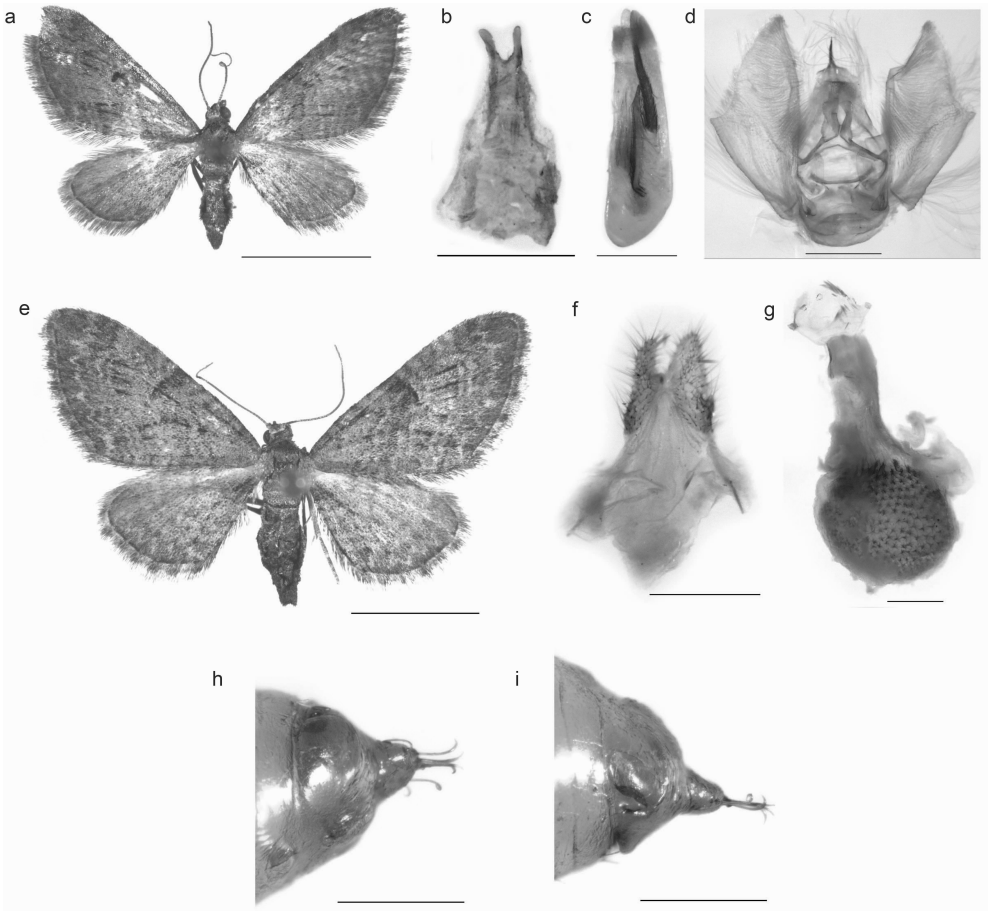

Figure 2. Morphology of *Eupithecia pusillata.* a) male, dorsal view, b) male, 8th sternite, c) male, aedeagus, d) male, genital capsule e) female, dorsal view, f) female, ovipositor, g) female, bursa copulatrix, h) pupa, terminal segment, dorsal view, i) pupa, terminal segment, lateral view. Scale bars: a, e = 5 mm; b–d, f–i = 0.5 mm. A colour version of this figure is available from Dr. Lee Humble.

Distribution and Habitat: In its native European range, the nominate subspecies is widely distributed from southern Europe, its range extends to the Mediterranean from eastern Spain to mainland Greece and Romania, then extends north and west across northern Ukraine into western Russia. With the exception of Corsica, it has not been recorded from the islands of the Mediterranean. To the north it is present in the British Isles, through central Europe, north to northern Scandinavia, and into western Russia across the southern Kola Peninsula (Skou 1986; Mironov 2003; Karsholt & van Nieukerken 2010). A disjunct population of *E. pusillata* is present in the Caucasus Mountains (Mironov 2003). In Asia, its range extends across Russia from Sahkalin through Siberia, the Altai and Caucasus regions (Skou 1986). The subspecies *E. pusillata scoriata* Staudinger, 1857 has been recorded only from Iceland and southwestern Greenland (Mironov 2003). Mironov et al. (2008) recently described a third subspecies, *E. pusillata kashmirica* Mironov and Ratzel from the Himalayas. In natural settings, *E. pusillata* can be found in heaths, forest edges, rocky cliffs, and similar habitats where the primary host grows. In urban areas, it can be common in gar-

dens. It is known from sea level up to approximately 2,500 m elevation in the Sierra Nevada (Spain) and the Alps (Switzerland) (Weigt 1993; Mironov 2003).

Life History and Notes: The following data are based on European populations, and it is expected that flight times, voltinism and larval hosts will be similar in North America, should extant populations be discovered. Univoltine, with larval stage from late April to mid-June and adult flight period from mid-July to late September (Skou 1986; Mironov 2003). As its common name implies, the primary host of *E. pusillata* is common juniper, *Juniperus communis* L. (Cupressaceae) (Skou 1986), of which it feeds on young needles and flowers. It is generally regarded as monophagous (Mironov 2003), although it has also been recorded feeding on Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco (Pinaceae) in France (Roques et al. 2006), where this North American tree is cultivated. The host of the subspecies *scoriata* and *kashmirica* is not known, but is presumed to also be *Juniperus*. *Eupithecia pusillata* overwinters in the egg stage and pupates in a loose web in the ground (Skou 1986). It is attacked by a variety of ichneumonid and braconid species listed in Mironov (2003). It is not known if other native or ornamental species of *Juniperus* are suitable hosts in British Columbia.

DISCUSSION

Eupithecia Curtis is a large genus with 1529 described species and subspecies (Scoble 1999; Scoble & Hausmann 2007), and about 160 species in North America (Powell and Opler 2009). The North American species were revised by McDunnough (1949), and the Canadian fauna was revised by Bolte (1990). *Eupithecia pusillata* is part of the *niphadophilata* species group, which includes two Nearctic and one Palearctic species (Bolte 1990), all feeding primarily on junipers (Skou 1986; Bolte 1990).

Although we currently have only two specimens of *Eupithecia pusillata* from North America, we can extract a great deal of information from the associated data documentation. First of all, the collections were made in urbanized Vancouver, BC, suggesting the species was introduced. The lack of records, particularly from inland BC (which is well-surveyed for macro-Lepidoptera), the Yukon Territory and Alaska, lead us to conclude that the species is not naturally Holarctic like some *Eupithecia* (see Skou 1986, Bolte 1990). Furthermore, the six *Eupithecia* species considered Holarctic all show at least 1% COI sequence divergence (data not shown) indicative of separation in the Pleistocene. The absence of additional records also suggests that there has not been substantial spread beyond the point of introduction. Secondly, the locality of the first collection (Mt. Burke), the number of individuals recorded (ten), and the damage observations in the FIDS record, all indicate that there was an established *E. pusillata* population in BC in 1976 (but note this is the only FIDS record of a *Eupithecia* on juniper from greater Vancouver). And lastly, the 1986 collection from North Vancouver suggests that the population has persisted, or it did so for at least a decade. Subsequent surveys, initially in the Vancouver area, are required to determine the contemporary status of this species.

The excellent documentation of FIDS that enabled inferences about the status of *E. pusillata* is unfortunately a relict of the past; the program ceased in 1996 after almost 50 years of operation due to budgetary cut-backs (Van Sickle et al. 2001). Programs such as this, based on surveying or inventorying diversity, are simultaneously a) a tremendous resource for managers, foresters and scientists, and b) reliant on tremendous resources themselves particularly in terms of highly qualified personnel (e.g. Marshall et al. 1994). The present case illustrates the value of these long-term, well-documented biological surveys, but these programs are often hindered by the

necessity to rear immatures to allow the diagnosis of species. Just as DNA barcoding makes an invaluable tool for biosurveillance (Floyd et al. 2010), it could likewise assist any regional or national biomonitoring program of similar scope to FIDS. Barcoding could not only identify immature stages (Ahrens et al. 2007) making rearing nonobligatory, it could also identify the plant meal of gut contents (Miller et al. 2007), identify parasitoids (Rougerie et al. in press), and trace complex food webs (Sheppard et al. 2004; Smith et al. in press). Decreasing costs and increasing capabilities of sequencing (e.g. Shokralla et al. 2010) are certain to make species diagnosis in this form time- and cost-effective. Furthermore, most years of the FIDS program predated electronic databases, so it would also be better served by modern and online relational databases such as BOLD (Ratnasingham and Hebert 2007). With DNA barcoding in place, a resource similar to FIDS could once again be realized, and without having to expend substantial resources as a cost. It would also, without question, speed the time of non-indigenous species detection—from years (34 in the case of *E. pusillata*) to days.

ACKNOWLEDGEMENTS

We are indebted to Axel Hausmann (Zoological State Collection Munich) and Marko Mutanen (University of Oulu) for allowing us access to the Eurasian sequences. We also wish to thank Stephanie Kirk for the specimen imaging, the staff at the Canadian Centre for DNA Barcoding for their assistance with genetic analysis, and Sava Barudzija (Natural Resources Canada, Canadian Forest Service, Fredericton, NB) for extraction of historical FIDS records. Rene Alfaro, Isabel Leal and two anonymous reviewers provided valuable comments on the manuscript. Funding was provided by a Forest Investment Account - Forest Science Program Student Grant and NSERC Graduate Scholarship (to JRD). Molecular analyses were supported by the Canadian Barcode of Life Network from Genome Canada through the Ontario Genomics Institute, NSERC (to LMH), and other sponsors listed at www.BOLNET.ca.

REFERENCES

Ahrens, D., M.T. Monaghan and A.P. Vogler. 2007. DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (Coleoptera: Scarabaeidae). Molecular Phylogenetics and Evolution 44: 436–449.

Armstrong, K.F. 2010. DNA barcoding: a new module in New Zealand's plant biosecurity diagnostic toolbox. EPPO Bulletin 40: 91–100.

Armstrong, K.F. and S.L. Ball. 2005. DNA barcodes for biosecurity: invasive species identification. Philosophical Transactions of the Royal Society B: Biological Sciences 360: 1813–1823.

Ball, S.L. and K.F. Armstrong. 2006. DNA barcodes for insect pest identification: a test case with tussock moths (Lepidoptera: Lymantriidae). Canadian Journal of Forest Research 36: 337–350.

Barr, N.B. 2009. Pathway analysis of *Ceratitis capitata* (Diptera: Tephritidae) using mitochondrial DNA. Journal of Economic Entomology 102: 401–411.

Bolte, K.B. 1990. Guide to the Geometridae of Canada (Lepidoptera). VI. Subfamily Larentinae. I. Revision of the genus *Eupithecia*. Memoirs of the Entomological Society of Canada 151: 1–253.

deWaard, J.R., J.-F. Landry, B.C. Schmidt, J. Derhousoff, J.A. McLean and L.M. Humble. 2009. In the dark in a large urban park: DNA barcodes illuminate cryptic and introduced moth species. Biodiversity and Conservation 18: 3825–3839.

Floyd, R., J. Lima, J.R. deWaard, L.M. Humble and R.H. Hanner. 2010. Common goals: incorporating DNA barcoding into international protocols for identification of arthropod pests. Biological Invasions 12: 2947–2954.

Gilligan, T.M. and M.E. Epstein. 2009. LBAM ID, Tools for diagnosing light brown apple moth and related western U. S. leafrollers (Tortricidae: Archipini). Colorado State Univeristy, California Department of Food and Agriculture, and Center for Plant Health Science and Technology, USDA, APHIS, PPQ.

[online]. Available from http://keys.lucidcentral.org/keys/v3/LBAM/about.html [accessed 30 August 2010].

Humble, L.M., J.R. deWaard, and M. Quinn. 2009. Delayed recognition of the European poplar shoot borer, *Gypsonoma aceriana* (Duponchel) (Lepidoptera: Tortricidae) in Canada. Journal of the Entomological Society of British Columbia 106: 61–70.

Karsholt, O. and E.J. van Nieukerken. 2010. Fauna Europaea: Geometridae. Fauna Europaea version 2.2 [online]. Available from http://www.faunaeur.org [accessed 24 August 2010].

Lafontaine, J.D. 2004. Noctuoidea: Noctuidae (part), Noctuinae (part – Agrotini). Pages 1-385. *In* The moths of North America, fasc. 27.1. *Edited by* Hodges, R.W. Wedge Entomological Research Foundation, Washington, DC.

Marshall, S.A., R.S. Anderson, R.E. Roughley, V. Behan-Pelletier and H.V. Danks. 1994. Terrestrial arthropod biodiversity: planning a study and recommended sampling techniques. A brief. Bulletin of the Entomological Society of Canada 26: Supplement. 33 pp.

McDunnough, J.H. 1949. Revision of the North American species of the genus *Eupithecia* (Lepidoptera, Geometridae). Bulletin of the American Museum of Natural History 93: 533-728.

Miller, M.A., G.C. Müller, V.D. Kravchenko, A. Junnila, K.K. Vernon, C.D. Matheson and A. Hausmann. 2007. DNA-based identification of Lepidoptera larvae and plant meals from their gut content. Russian Entomological Journal 15: 427–432.

Mironov, V. 2003. Larentiinae II (Perizomini and Eupitheciini). *In* A. Hausmann (ed.): The Geometrid Moths of Europe 4: 1–463.

Mironov, V.G., A.C. Galsworthy and U. Ratzel. 2008. A survey of the *Eupithecia* fauna (Lepidoptera, Geometridae) of the Western Himalayas: Part II. Transactions of the Lepidopterological Society of Japan 59: 117–143.

Nadel, R.L., B. Slippers, M.C. Scholes, S.A. Lawson, A.E. Noack, C.F. Wilcken, J.P. Bouvet and M.J. Wingfield. 2010. DNA bar-coding reveals source and patterns of *Thaumastocoris peregrinus* invasions in South Africa and South America. Biological Invasions 12: 1067–1077.

Powell, J.A. and P.A. Opler. 2009. Moths of western North America. University of California Press, Berkeley, 369 pp.

Ratnasingham, S. and P.D.N. Hebert. 2007. BOLD: the barcode of life data system (http://www.barcodinglife.org). Molecular Ecology Notes 7: 355–364.

Roques, A., M.A. Auger-Rozenberg and S. Boivin. 2006. A lack of native congeners may limit colonization of introduced conifers by indigenous insects in Europe. Canadian Journal of Forest Research 36: 299–313.

Rougerie, R., M.A. Smith, J. Fernandez-Triana, C. Lopez-Vaamonde, and P.D.N. Hebert. Molecular analysis of parasitoid linkages (MAPL): gut contents of adult parasitoid wasps reveal larval host. Molecular Ecology. In press

Scheffer, S.J., M.L. Lewis, and R.C. Joshi. 2006. DNA barcoding applied to invasive leafminers (Diptera: Agromyzidae) in the Philippines. Annals of the Entomological Society of America 99: 204–210.

Scoble, M.J. (Ed) 1999. Geometrid moths of the world: a catalogue (Lepidoptera: Geometridae). Natural History Museum, London, UK and CSIRO Publishers, Collingwood, Australia, 1016 pp.

Scoble, M.J. and A. Hausmann. 2007. Online list of valid and available names of the Geometridae of the World [online]. Available from http://www.lepbarcoding.org/geometridae/species_checklists.php [accessed 27 August 2010].

Sheppard, S.K., M.L. Henneman, J. Memmott and W.O. Symondson. 2004. Infiltration by alien predators into invertebrate food-webs in Hawaii: a molecular approach. Molecular Ecology 13: 2077–2088.

Shokralla, S., G.A.C. Singer and M. Hajibabaei. 2010. Direct PCR amplification and sequencing of specimens' DNA from preservative ethanol. Biotechniques 48: 305–306.

Siddall, M.E. and R.B. Budinoff. 2005. DNA-barcoding evidence for widespread introductions of a leech from the South American *Helobdella triserialis* complex. Conservation Genetics 6: 467–472.

Simonsen, T.J., R.L. Brown and F.A.H. Sperling. 2008. Tracing an invasion: phylogeography of *Cactoblastis cactorum* (Lepidoptera: Pyralidae) in the United States based on mitochondrial DNA. Annals of the Entomological Society of America 101: 899–905.

Skou, P. 1986. The Geometroid Moths of North Europe. Scandinavian Science Press. 348 p.

Smith, M.A., E.S. Eveleigh, K.S. McCann, P.C. McCarthy and K.I. Van Rooyen. Barcoding a quantified food web: crypsis, concepts, ecology and hypotheses. PLoS ONE. In press.

Van Sickle, A., R.L. Fiddick and C.S. Wood. 2001. The forest insect and disease survey in the Pacific Region. Journal of the Entomological Society of British Columbia 98: 169–176.

Weigt, H.-J. 1993. Die Blütenspanner Mitteleuropas (Lepidoptera, Geometridae: Eupitheciini). Teil 5. - Dortmunder Beitrage zur Landeskunde 27: 5–108.

Wilson, A.D. and N.M. Schiff. 2010. Identification of *Sirex noctilio* and native North American woodwasp larvae using DNA barcodes. Journal of Entomology 7: 60–79.