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ORIGINAL PAPER

In the dark in a large urban park: DNA barcodes illuminate cryptic and introduced moth species

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Abstract To facilitate future assessments of diversity following disturbance events, we conducted a first level inventory of nocturnal Lepidoptera in Stanley Park, Vancouver, Canada. To aid the considerable task, we employed high-throughput DNA barcoding for the rough sorting of all material and for tentative species identifications, where possible. We report the preliminary species list of 190, the detection of four new exotic species (*Argyresthia pruniella*, *Dichelia histrionana*, *Paraswammerdamia lutarea*, and *Prays fraxinella*), and the potential discovery of two cryptic species. We describe the magnitude of assistance that barcoding presents for faunal inventories, from reducing specialist time to facilitating the detection of native and exotic species at low density.

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Introduction

The biodiversity inventory, in all but a few taxa, and at all but the shallowest levels, is inherently a formidable task. For the vast majority of terrestrial arthropods, enumerating and naming all residents of a community or assemblage is onerous and requires a substantial investment of resources. Large samples, damaged specimens, immature stages—all of these may be commonplace when conducting surveys of hyper-diverse groups, and all of these can provide a substantial barrier to the cornerstone of biodiversity studies: the accurate diagnosis of species. To compound this problem, the lack of trained professional systematists and taxonomists, and the subsequent lack of usable keys and modern nomenclature (Gotelli 2004), threatens the extinction of the faunal inventory for all but a few well-known groups (e.g. butterflies, tiger beetles, and dragonflies).

The establishment of DNA barcoding (Hebert et al. 2003) holds significant promise to overcome some of the obstacles of biodiversity inventories. In particular, barcoding can transform the often lengthy and tedious chore of identifying specimens to a rapid, accurate and unbiased task (Janzen et al. 2005; Smith et al. 2005). Even when a comprehensive database is unavailable, it can still assist with the rough sorting of specimens, guide morphological determinations, and populate the database for future surveys. This may free time for specialists and highly qualified personnel, fostering improved inventories and repeated monitoring.

In the wake of the three windstorms of the 2006–2007 winter that caused significant destruction in Stanley Park, Vancouver, Canada (see Vancouver Park Board 2007), it became apparent that the biodiversity data necessary to appraise the effects were lacking. Furthermore, concern mounted over the forest's increased susceptibility to exotic species, particularly within various terrestrial arthropod groups. To remedy this, baseline surveys and monitoring projects in several insect groups were established (McLean et al. 2009a, b), including the initiation of a first level inventory of the nocturnal Lepidoptera assemblage. To aid with the latter, we conducted high-throughput DNA barcoding for rough sorting of all material and for assigning tentative species identifications where possible. We evaluate this 'wedding' of barcoding and inventories (Janzen et al. 2005) and its effectiveness for the initial screening for a faunal inventory in a hyper-diverse group, as well as its ability to flag exotic and cryptic species.

Materials and methods

Specimens were collected by mercury-vapour light at two sites in Stanley Park, Vancouver, Canada: the west side of the park, near the 'Hollow Tree' (49.306N 123.153W, 13 m) and on the eastern half, near the Vancouver Aquarium (49.301N 123.128W, 52 m). The sampling effort consisted of eight collections made between May and August 2007 beginning at dusk for roughly 5 h. Specimens were hand-collected live and killed by freezing or ammonium hydroxide just prior to mounting and spreading (Landry and Landry 1994). A synoptic collection was made for each night, retaining no more than five individuals per morphospecies. Specimens were labelled, photographed and all collateral data

and images were uploaded to the project 'Lepidoptera of Stanley Park' (LBCS) in the Barcode of Life Database (BOLD) (Ratnasingham and Hebert 2007).

From each specimen, one or two legs were removed and stored in an individual tube of a 96-tube sample box (Matrix Technologies). Subsequent analysis followed standard high-throughput DNA barcoding methods (Hajibabaei et al. 2005; deWaard et al. 2008a) with a few modifications. Tissue was placed in a 96-well plate of proteinase K lysis buffer and incubated for roughly 18 h. The lysate was then processed following the glass-fibre protocol of Ivanova et al. (2006) on a Biomek FX^P liquid handler (Beckman Coulter). For PCR amplification, 2 μl of DNA extract was added to each well of a premade PCR plate stored at −20°C and containing 2 μl of H_2O, 6.25 μl of 10% trehalose, 1.25 μl of 10× buffer, 0.625 μl of 50 mM $MgCl_2$, 0.0625 μl of 10 mM dNTPs, 0.06 μl of Platinum Taq polymerase (Invitrogen) and 0.125 μl of each of the 10 μM primers LepF1 and LepR1 (Hebert et al. 2004). The thermocycling conditions consisted of an initial denaturation at 94°C for 1 min, five cycles of 94°C for 30 s, annealing at 45°C for 40 s, and extension at 72°C for 1 min, followed by 35 cycles of 94°C for 30 s, 51°C for 40 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR reactions were visualized with the E-Gel 96 agarose electrophoresis system (Invitrogen) before performing the sequencing reactions, again in premade and frozen plates. Both the forward and reverse direction plates contained 0.25 μl of Dye terminator mix v3.1 (Applied Biosystems), 1.875 μl of 5× sequencing buffer, 5 μl of 10% trehalose, and 1 μl of the respective 10 μM PCR primer. Sequencing reactions were run at an initial denaturation at 96°C for 2 min, followed by 30 cycles of 96°C for 30 s, annealing at 55°C for 15 s, and extension at 60°C for 4 min. The reactions were purified using the CleanSEQ system (Agencourt Bioscience) on a Biomek FX^P liquid handler before being run on a 3730XL DNA Analyzer (Applied Biosystems), all following manufacturer's instructions. Electropherograms were edited and aligned in Seqscape v. 2.5 (Applied Biosystems) and the resultant sequences were uploaded to BOLD.

The identification engine of BOLD (BOLD-ID) was used for assigning tentative identifications, where possible, for all sequences. The reference barcode database for Lepidoptera used by BOLD-ID is continually validated by specialists ensuring accurate determinations (see http://www.lepbarcoding.org/campaign_nth_am.php for details). An identification was considered definitive if a similarity score of 98.5–100% was obtained, and the match was with a single monophyletic species. These barcode-assigned determinations were subsequently confirmed morphologically with comparison to reference specimens in regional insect collections. Cases that were not assigned a definitive identification were keyed to species, performing genitalic dissections where necessary. All specimens were deposited in the Royal British Columbia Museum, Victoria, BC (RBCM), Pacific Forestry Centre, Canadian Forest Service, Victoria, BC (PFCA), the Spencer Entomological Museum, University of British Columbia, Vancouver, BC (UBCZ), and the Canadian National Collection of Insects, Agriculture and Agri-Food Canada, Ottawa, ON (CNC) (Supplementary Table A1).

In order to explore the completeness of our inventory, we calculated accumulation curves using incidence-based methods. Firstly, we used the method of Colwell et al. (2004) and Mao et al. (2005) to interpolate the curve for expected total and singleton species caught; the proportion of singletons can be indicative of the completeness of the census (Longino et al. 2002). Secondly, we calculated two robust (e.g. Chazdon et al. 1998; Summerville and Crist 2005) nonparametric estimators of species richness, the ICE (Lee and Chao 1994) and Chao 2 (Chao 1987) estimators. The program EstimateS v. 8.0 (Colwell 2006) was employed for all analyses, computing curves as the mean of 1,000 randomized species accumulation curves without replacement.

Fig. 1 Neighbour-joining tree of the nocturnal Lepidoptera collected in Stanley Park, Vancouver, Canada ▶ in 2007. Two specimens are excluded (UBC-2007-0320 and UBC-2007-0322) due to short COI sequence length. The number of specimens collapsed into a single node is given in *parentheses* after the taxon name

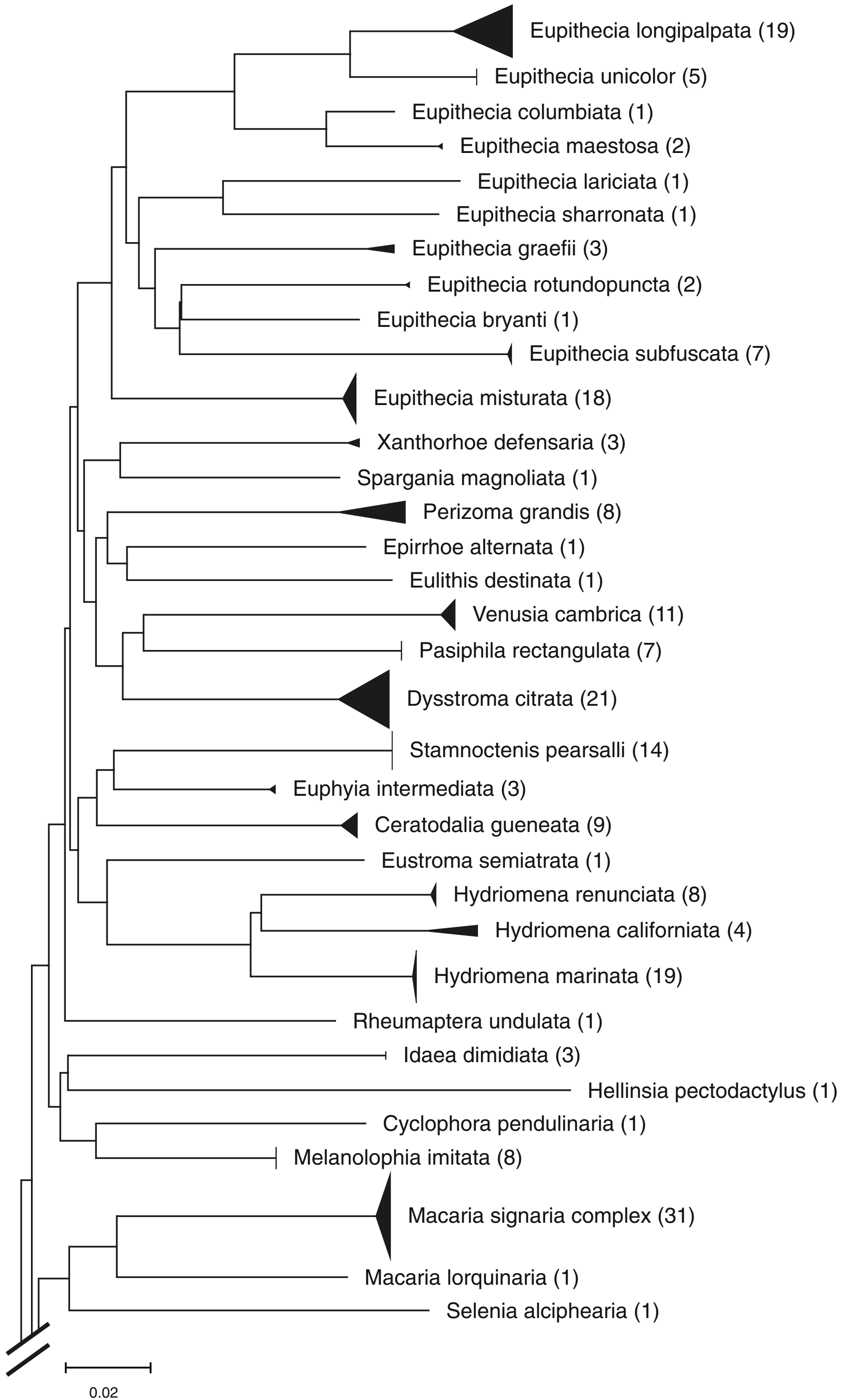
Results

The 8 collections ranged from 41 to 225 moths per night for a total of 925 specimens. The first attempt at barcoding the samples resulted in 912 being successfully amplified and sequenced; the sequences for the remaining 13 were obtained by simply repeating the procedures from extraction onwards. A total of 895 specimens provided the full 658 base pair (bp) barcode region and the remaining samples ranged from 119 to 646 bp in sequence length. The sequences and electropherograms are publicly available on BOLD and GenBank (accession nos. FJ412108–FJ413032; Supplementary Table A1), while DNA extracts are archived at −80°C at the Canadian Centre for DNA Barcoding in Guelph, Canada to allow validation and facilitate future biodiversity and genomic research (Hanner and Gregory 2007).

The aligned sequences resulted in ~190 clusters with <3% sequence divergence (Fig. 1). BOLD-ID unequivocally assigned 124 of these to species based on the complete BOLD database (Supplementary Table A2), and a further 61 were tentatively placed to genus. The length of sequence generated was not inhibitive, as demonstrated previously (Hajibabaei et al. 2006a); the two shortest sequences (UBC-2007-0320—119 bp and UBC-2007-0322—172 bp) were both reliably assigned to *Batia lunaris*. For the barcode assigned identifications, three generic assignments were later corrected after morphological examination (UBC-2007-0481–0482 assigned to *Pandemis* sp., corrected as *Argyrotaenia dorsalana*; UBC-2007-0180–0182, 0261–265, and 0485–0489: assigned to *Clepsis* sp., corrected as *Argyrotaenia provana*; and UBC-2007-0871 assigned to *Battaristis* sp., corrected as *Coleotechnites* sp. nr. *coniferella*). For the 66 clusters not assigned to species or genus by BOLD-ID, 25 were identified by brief comparison with reference material, and the final 41 were identified following genitalic preparation.

Following the barcoding and morphological examination, 190 species (or sub-generic taxa) representing 21 families were determined (Supplementary Table A2). Due to incomplete taxonomy in some groups, particularly in the Microlepidoptera, 15 taxa have been given interim names (e.g. *Acleris* JFL01, *Macaria signaria* complex, *Homosetia* n. sp. nr. *costisignella*). Represented as a taxon-ID tree from BOLD (Fig. 1), it is apparent that barcodes clearly delimit all species. The mean divergence between congeneric species is 9.50% (range = 1.541–15.327%, SE = 0.061%) and within species is 0.258% (range = 0–3.596%, SE = 0.007%) (Fig. 2). If an arbitrary threshold of 3% (Kimura 2-parameter distance) is set (Hebert et al. 2003), all but 2 species pairs can be differentiated (*Dioryctria pseudotsugella*/*reniculelloides* and *Chionodes periculella*/*abella*), and only 2 species display intraspecific divergences >3% (*Perizoma grandis* and *Dasypyga alternosquamella*) that would potentially inflate the estimation of species number.

As is nearly universal in biodiversity inventories, a high prevalence of dominance and rarity was revealed by the identified collection. Roughly two-thirds of the species and individuals collected belong to 3 dominant families: the Geometridae (47 spp./338 specimens), Tortricidae (37/189), and Noctuidae (33/102). On the other end of the spectrum, 93 species were collected in a single sample (uniques). Similarly, 29 species are represented by only two specimens (doubletons) and 71 are represented by a single individual (singletons). This large proportion of rare individuals indicates that the inventory remains incomplete.



Eupithecia longipalpata (19)
Eupithecia unicolor (5)
Eupithecia columbiata (1)
Eupithecia maestosa (2)
Eupithecia lariciata (1)
Eupithecia sharronata (1)
Eupithecia graefii (3)
Eupithecia rotundopuncta (2)
Eupithecia bryanti (1)
Eupithecia subfuscata (7)
Eupithecia misturata (18)
Xanthorhoe defensaria (3)
Spargania magnoliata (1)
Perizoma grandis (8)
Epirrhoe alternata (1)
Eulithis destinata (1)
Venusia cambrica (11)
Pasiphila rectangulata (7)
Dysstroma citrata (21)
Stamnoctenis pearsalli (14)
Euphyia intermediata (3)
Ceratodalia gueneata (9)
Eustroma semiatrata (1)
Hydriomena renunciata (8)
Hydriomena californiata (4)
Hydriomena marinata (19)
Rheumaptera undulata (1)
Idaea dimidiata (3)
Hellinsia pectodactylus (1)
Cyclophora pendulinaria (1)
Melanolophia imitata (8)
Macaria signaria complex (31)
Macaria lorquinaria (1)
Selenia alciphearia (1)
0.02

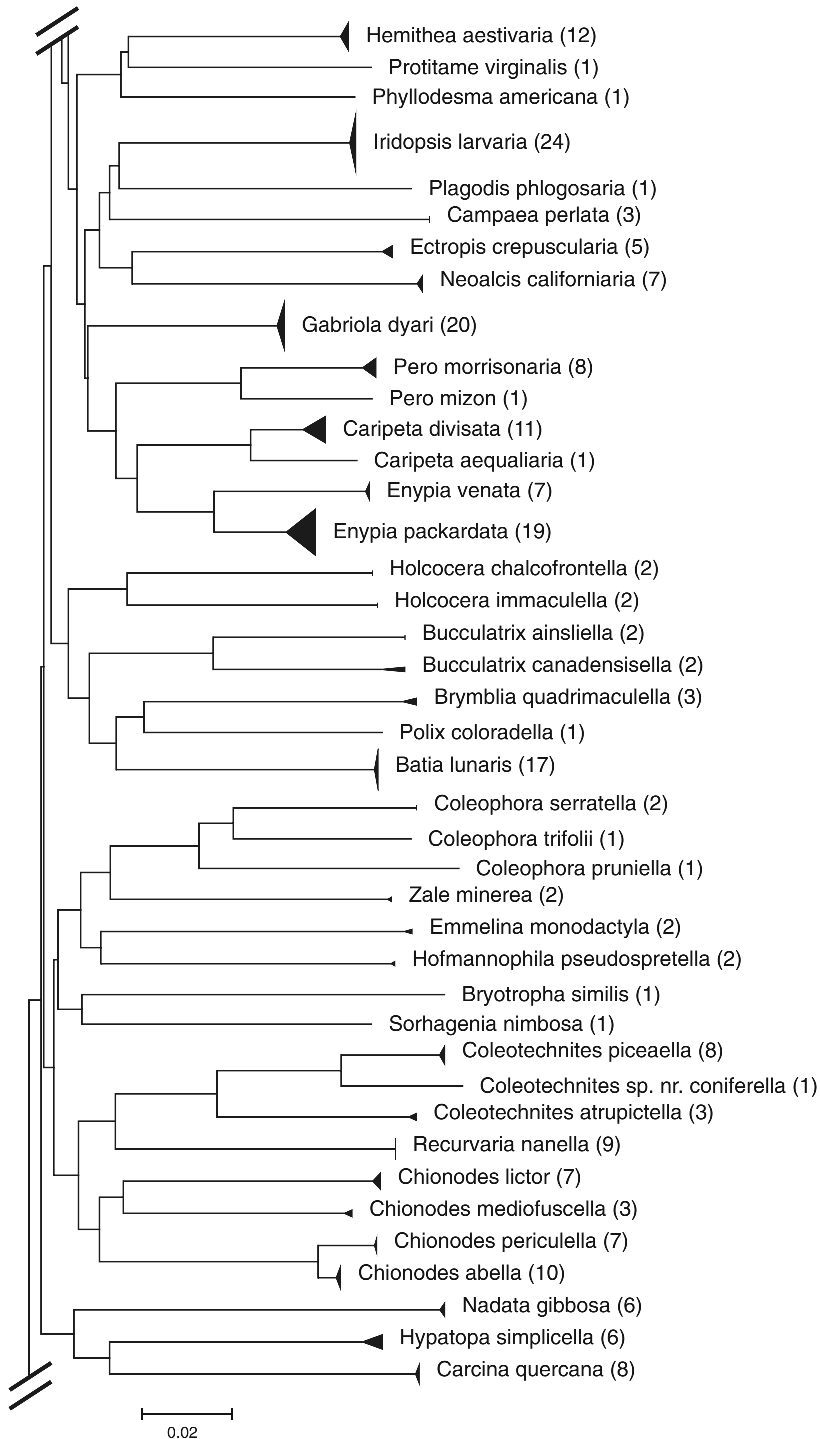
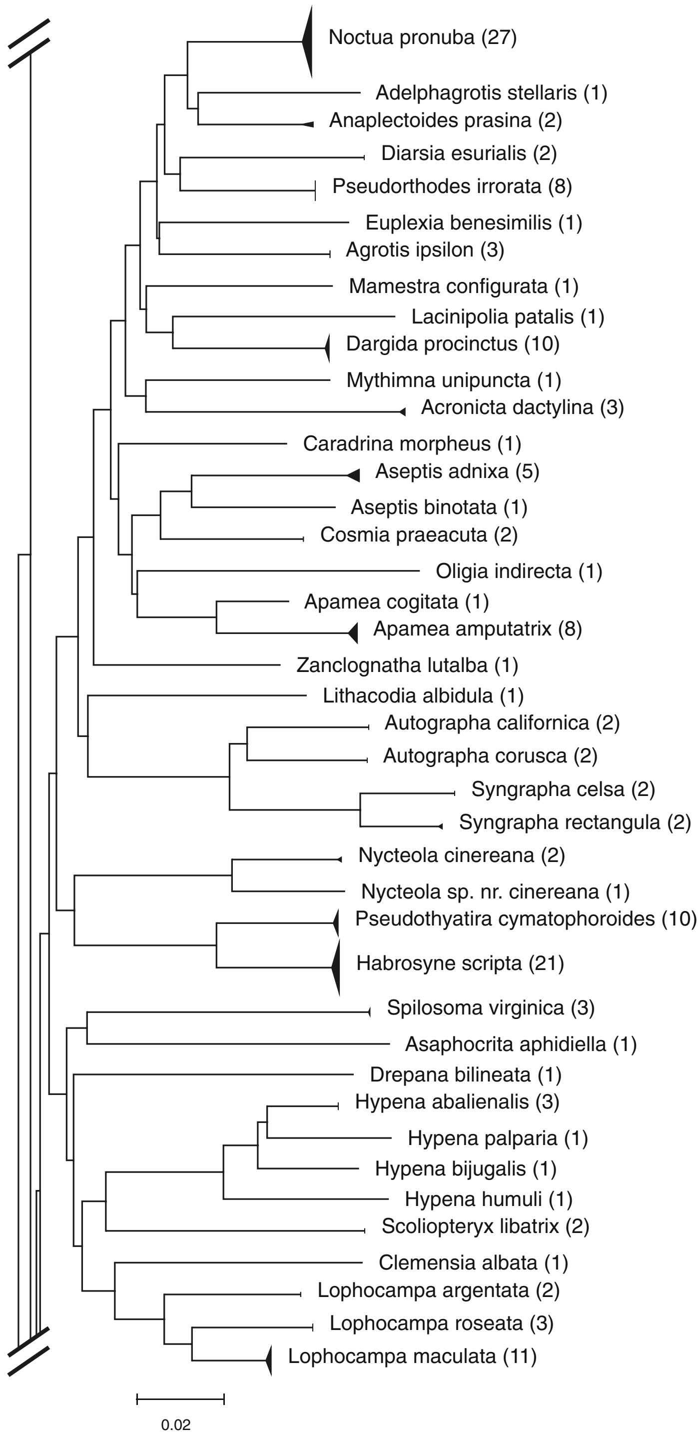


Fig. 1 continued



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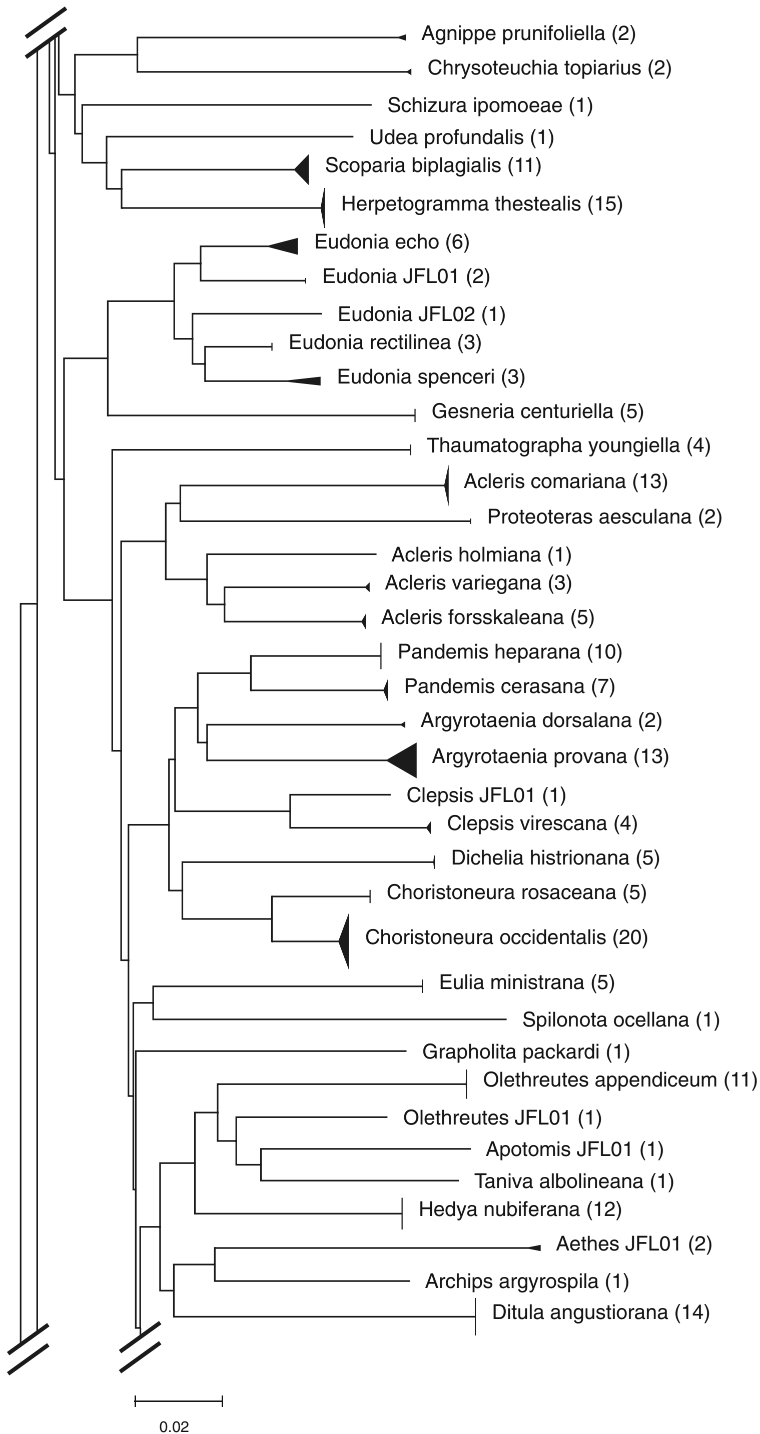

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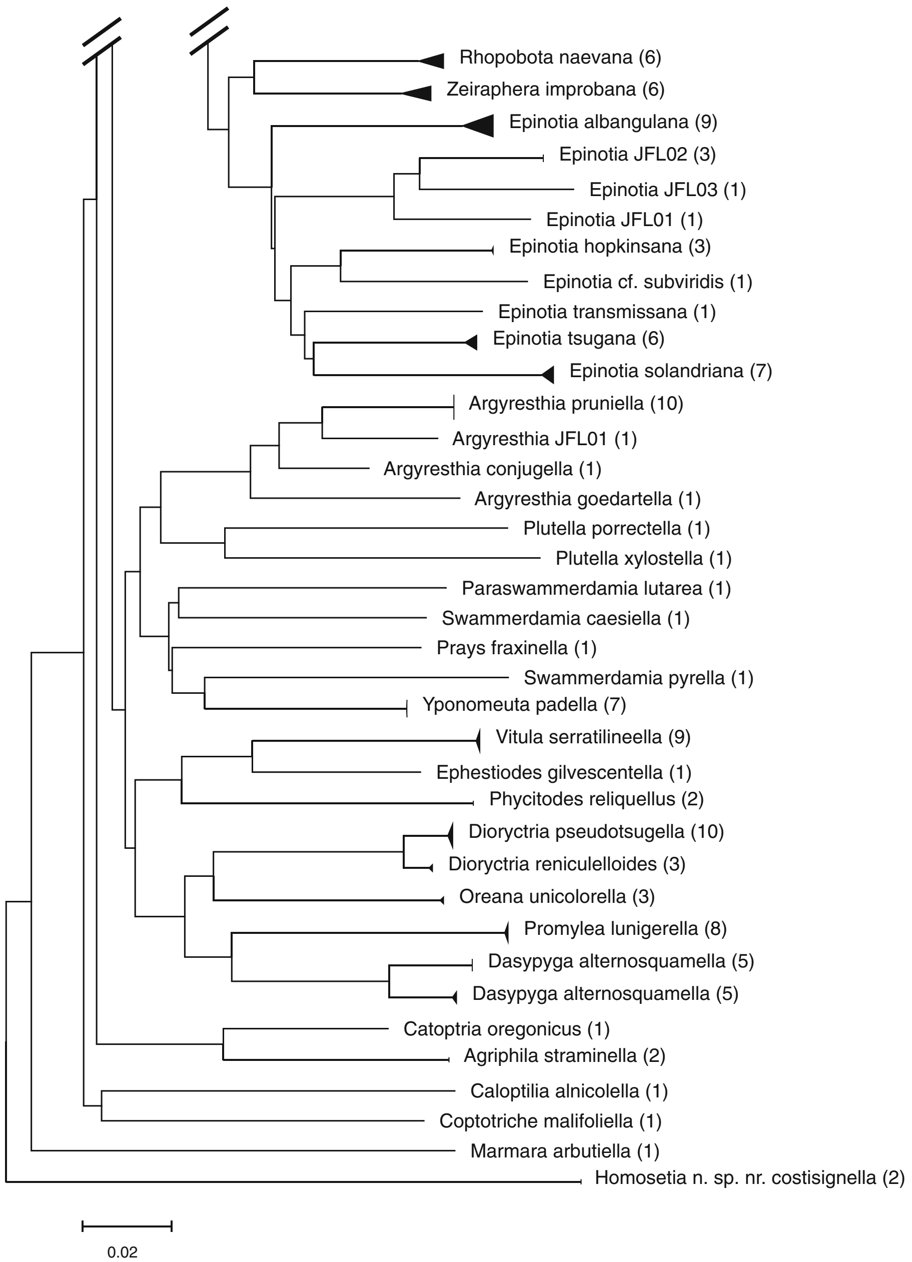

Fig. 1 continued

There is also a fairly high incidence of nonindigenous species; 31 introduced species that have evidently or presumably established were identified in the collection. Among these, three species represent new records for North America (*Argyresthia pruniella*,

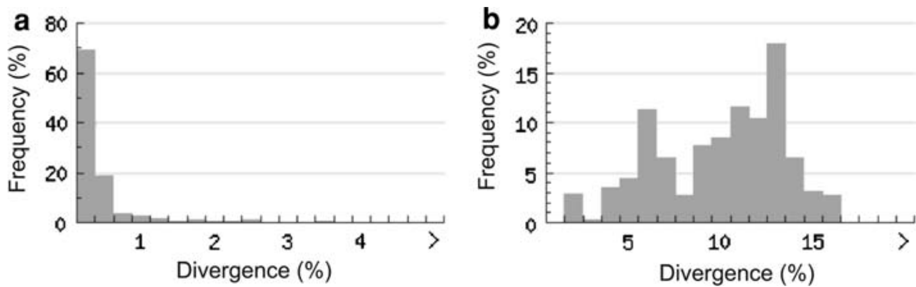

Fig. 2 Distance summary of the 925 COI barcodes generated for the Stanley Park moth specimens: **a** histogram of intraspecific divergences for the 190 species (or subgeneric taxa), and **b** histogram of congeneric distances for 441 individuals (3,175 comparisons)

Table 1 Descriptions for four introduced moth species discovered in Stanley Park, Vancouver, Canada in 2007

Taxonomy	Host plant(s)	Distribution (outside BC)	References
Paraswammerdamia lutarea (Haworth, 1828)	Maloideae (cottoneaster, hawthorn, rowan)	Europe—widespread	Karsholt and Razowski (1996)
Prays fraxinella (Donovan, 1793)	*Fraxinus excelsior* (ash)	Europe—widespread, Newfoundland[a]	Karsholt and Razowski (1996)
Dichelia histrionana (Frölich, 1828)	*Picea* (spruce) and *Abies* (fir)	Europe including Scandinavia & British Isles, E to Caucasus	Sterling and Ashby (2006)
Argyresthia pruniella (Clerck, 1759)	Rosaceae (stone-fruit cultures)	Europe and Asia Minor, Nova Scotia[b]	Agassiz (1996) and Karsholt and Razowski (1996)

[a] One unpublished record in the CNC from 1975 reared from ash

[b] Following its detection in BC, a series from Nova Scotia from the 1960s was discovered in the USNM (Washington)

Dichelia histrionana, and *Paraswammerdamia lutarea*) and one species has not been previously detected in BC (*Prays fraxinella*) (Table 1). In the latter case, BOLD-ID indicated a close match between the single specimen (UBC-2007-0308) and two Eurasian *Prays* spp. (*P. oleae*—Spain; *P. epsilon*—South Korea) suggesting that the (previously) single specimen of *P. fraxinella* from Newfoundland in 1975 was no longer the only North American record. In addition to these revelations, two instances of potentially cryptic species were discovered: *Dasypyga alternosquamella* (3.27–3.60% divergence between clusters) and *Nycteola* sp. nr. *cinereana* (4.56–4.72% divergent from *N. cinereana*).

The incidence-based accumulation curves estimated indicate that the number of species may be starting to approach an asymptote (Fig. 3). The two nonparametric estimators of total species richness (including the unsampled portion) intersected at a near-identical value: Chao 2 = 307, ICE = 309. This represents a conservative, minimum estimate of richness (Longino et al. 2002), suggesting that the total nocturnal species present in the two collection localities may be over 60% higher than measured.

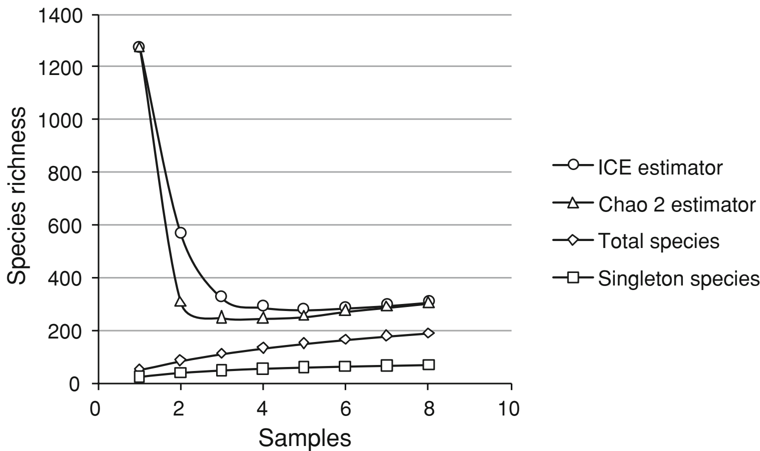

Fig. 3 Observed species richness, observed singletons and ICE and Chao 2 estimators as a function of sampling effort (collection nights) for the inventory of nocturnal Lepidoptera in Stanley Park, Vancouver, Canada conducted in 2007

Discussion

Barcode recovery and success

The DNA barcoding of the fresh specimens was straightforward and the recovery of a sufficient fragment of COI for identification was made for all specimens. Due to three reasons, a large proportion of the steps from specimen collection to identified material were completed by non-specialists. Firstly, limited training and supervision is required for a technician or parataxonomist to collect insect specimens in the field, roughly separate them into morphospecies, prepare the specimens for museum deposition, and sample them for DNA analysis. Furthermore, if trapping methods (e.g. UV light traps, pitfall traps, flight interception traps) are employed, and specimens are not sorted to morphospecies, the process becomes even further routine. Secondly, the laboratory protocols are now highly refined, rapid, and undemanding (Hajibabaei et al. 2005; deWaard et al. 2008a) and many steps can be automated where laboratory infrastructure permits (e.g. Ivanova et al. 2006). This allows technicians with minimal training and supervision to perform all necessary steps, and to complete them in small laboratory facilities. And lastly, the process of barcoding itself not only identifies specimens that already exist in the sequence database, but they also limit and guide the downstream work of specialists by sorting unidentified specimens into operational taxonomic units (OTUs) that require examination and may provide higher taxonomic assignments (e.g. genus). In the present study, 94% of individuals were placed to genus or better prior to a taxonomic specialist viewing the material.

The present study demonstrates not only the successful recovery of barcodes, but the clear utility of barcodes for differentiating species of Lepidoptera, particularly at a small regional scale. Although two species pairs display shallow inter-specific divergence and two species display deep intra-specific divergence, in all cases the barcode groups are distinct and monophyletic, and would not prevent a successful species assignment by COI. This perfect success rate is comparable to two recent studies on Lepidoptera, a 97.9% success rate in a tropical, regional study of 521 species (Hajibabaei et al. 2006b), and the 98.5% observed in a temperate, continental study of 1,327 species (Hebert et al. unpublished).

Inventory progress

Facilitated by DNA barcoding, a first level inventory of Stanley Park is now complete. While the list contains 190 species, several are without proper species epithets, and the nonparametric estimators suggest that a minimum of roughly 120 additional species remain to be sampled in the assemblage. This is undoubtedly a conservative estimate as collecting was limited to two sites and to some extent, an abbreviated season. Moreover, Grimble et al. (1992) conducted a comparable inventory in the Pacific Northwest region and tallied 383 nocturnal moth species. Therefore, several recommendations can be made for the inventory to move forward.

First of all, continued work on the 15 taxa with interim names should reveal their identity. Revisions for a few of the difficult groups are underway or completed (e.g. macarine geometrids—Ferguson 2008) and others are under examination as part of a continental campaign to barcode all North American Lepidoptera species (see http://www.lepbarcoding.org/campaign_nth_am.php). Secondly, with the asymptote in species number still not reached, it appears worthwhile to continue the barcode-assisted survey in the park. Future surveys would be best suited to record but not analyze the common and distinctive species (e.g. *Noctua pronuba*, *Gabriola dyari*, *Habrosyne scripta*, *Enypia packardata*) and increase the sampling of difficult groups (e.g. Tortricidae) where singletons may be hidden in assumed morphospecies (e.g. Clepsis JFL01 within UBC-2007-0854–UBC-2007-0858). Other sampling methods should be added, aimed at recovering species typically poorly attracted to light traps, particularly in the Microlepidoptera. Furthermore, sampling with light traps could be expanded to sample early spring and autumn flying species not sampled during this study, in addition to sampling unique microhabitats which likely harbour species not found at the two sampled sites. And lastly, specimens and species previously collected in the park and deposited in the regional and national insect collections can be verified and added to the checklist (e.g. Supplementary Tables A3 and A4). These recommendations would improve the taxonomic completeness of the survey, but the returns would diminish exponentially with increased effort. Moreover, for the purpose of producing a baseline on which to measure the effects of storms and other disturbances, the sampling design and effort are sufficient.

Highlights of the nocturnal lepidopteran fauna of Stanley Park

One interesting (and perhaps alarming) finding that can be drawn from the preliminary inventory is the high incidence of non-native species. Nearly one in every six species encountered is exotic. As incredible as this is, particular guilds might be even more skewed—Doganlar and Beirne (1978) found that introduced species comprised 5 of the 6 most common and 8 of the 11 total species of leafrollers in Vancouver, Canada. It is also rather distressing that we detected four new exotics in a single collecting season. This might be the product of the park's close proximity to shipping ports, the high diversity of ornamental plants and other non-native hosts, and the disturbed condition of the park following the windstorms that allowed the populations to increase to a level that could be detected. Another contributing factor could simply be the addition of DNA barcoding to the arsenal of detection (Armstrong and Ball 2005; Chown et al. 2008; deWaard et al. 2008b). Typically an introduced species persists at low population densities before becoming established (Tilman 2004) so it is expected that few if any individuals will be collected. Barcoding ensures these few individuals are not overlooked or lumped in with native species, by either matching an existing record in the database (e.g. deWaard et al.

2008b), receiving a generic (or higher level) assignment (e.g. *Prays fraxinella*, this study) or by merely flagging the individuals as unique and requiring further scrutiny.

The inventory has also brought another interesting finding to light—two instances that might represent previously overlooked species. The first case, *Nycteola* sp. nr. *cinereana* is nearly 5% divergent from the typical 'form' of *N. cinereana* Neumoegen & Dyar and only ~1.5% divergent from one of the two forms of this species complex in Colorado (J. D. Lafontaine, personal communication). The slight colouration and size differences are not coupled with genitalic variation, nor are they in the two forms studied here. The lack of genitalic differences does not necessarily suggest a single species, but it does indicate that a single species with an ancient COI polymorphism is a viable hypothesis that requires further study. Similarly, the second split representing a potentially cryptic species, that in *Dasypyga alternosquamella* Ragonot, 1887, does not demonstrate noticeable genitalic differences between the two distinct COI groups. Interestingly, Heinrich (1956) reports that when Ragonot described *D. alternosquamella*, he also described with it a "variety" (considered a subspecies by the International Code of Zoological Nomenclature) that he called *D. alternosquamella stictophorella*. The difference was a minor one in the forewing pattern which Heinrich considered a mere individual variant. Pending further examination, it remains to be seen whether one of the groups represents Ragonot's variety, a new species, or a COI polymorphism.

Conclusions

Biodiversity inventories must be rapid, reliable, and inexpensive (Coddington et al. 1996) but this ideal remains elusive for terrestrial arthropods. This study has demonstrated the accuracy and speed that DNA barcoding can contribute, as well as its potential for increased sensitivity for invasive and cryptic species detection, particularly at low densities. In light of the dropping costs of DNA barcoding and the emergence of new technologies (Hajibabaei et al. 2007), incorporating genetic methods into faunal inventories will soon be more cost and time-effective than current morphological methods.

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