Syst. Biol. 0(0):1–16, 2020
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DOI:10.1093/sysbio/syaa014

Longer is Not Always Better: Optimizing Barcode Length for Large-Scale Species Discovery and Identification

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Received 24 April 2019; reviews returned 20 January 2020; accepted 29 January 2020
Associate Editor: Jason Bond

Abstract.—New techniques for the species-level sorting of millions of specimens are needed in order to accelerate species discovery, determine how many species live on earth, and develop efficient biomonitoring techniques. These sorting methods should be reliable, scalable, and cost-effective, as well as being largely insensitive to low-quality genomic DNA, given that this is usually all that can be obtained from museum specimens. Mini-barcodes seem to satisfy these criteria, but it is unclear how well they perform for species-level sorting when compared with full-length barcodes. This is here tested based on 20 empirical data sets covering ca. 30,000 specimens (5500 species) and six clade-specific data sets from GenBank covering ca. 98,000 specimens (>20,000 species). All specimens in these data sets had full-length barcodes and had been sorted to species-level based on morphology. Mini-barcodes of different lengths and positions were obtained *in silico* from full-length barcodes using a sliding window approach (three windows: 100 bp, 200 bp, and 300 bp) and by excising nine mini-barcodes with established primers (length: 94–407 bp). We then tested whether barcode length and/or position reduces species-level congruence between morphospecies and molecular operational taxonomic units (mOTUs) that were obtained using three different species delimitation techniques (Poisson Tree Process, Automatic Barcode Gap Discovery, and Objective Clustering). Surprisingly, we find no significant differences in performance for both species- or specimen-level identification between full-length and mini-barcodes as long as they are of moderate length (>200 bp). Only very short mini-barcodes (<200 bp) perform poorly, especially when they are located near the 5′ end of the Folmer region. The mean congruence between morphospecies and mOTUs was ca. 75% for barcodes >200 bp and the congruent mOTUs contain ca. 75% of all specimens. Most conflict is caused by ca. 10% of the specimens that can be identified and should be targeted for re-examination in order to efficiently resolve conflict. Our study suggests that large-scale species discovery, identification, and metabarcoding can utilize mini-barcodes without any demonstrable loss of information compared to full-length barcodes. [DNA barcoding; metabarcoding; mini-barcodes; species discovery.]

The question of how many species live on earth has intrigued biologists for centuries, but we are nowhere close to having a robust answer. We do know that fewer than 2 million have been described and that there are an estimated 10–100 million multicellular species on the planet (Roskov et al. 2018). We also know that many are currently being extirpated by the "sixth mass extinction" (Ceballos et al. 2015; Sánchez-Bayo and Wyckhuys 2019), with potentially catastrophic consequences for the environment (Cafaro 2015). Monitoring, halting, and perhaps even reversing this process is frustrated by the "taxonomic impediment". This impediment is particularly severe for "invertebrates" that collectively contribute much of the animal biomass (e.g., Stork et al. 2015; Bar-On et al. 2018). Most biologists thus agree that there is a pressing need for accelerating species discovery and description. This very likely requires the development of new molecular sorting methods because the traditional approach involving parataxonomists or highly trained taxonomic experts is either too imprecise (Krell 2004) or too slow and expensive. Arguably, any replacement method based on DNA sequences should be accurate but also (1) rapid, (2) cost-effective, and (3) largely insensitive to DNA quality. These criteria are important because tackling the earth's biodiversity will likely require the processing of >500 million specimens, even under the very conservative assumption that there are only 10 million species (Stork 2018) and a new species is discovered with every 50 specimens processed. Cost-effectiveness is similarly important because millions of species are found in countries with limited funding and only basic research facilities. On the positive side, many species are already present in museum holdings as unsorted material, but such specimens often yield only degraded DNA (Cooper 1994). Therefore, methods that require DNA of high-quality and quantity are not likely to be suitable for large-scale species discovery in invertebrates.

High-Throughput Species Discovery with Barcodes

Conceptually, species discovery and description can be broken up into three steps. The first is obtaining specimens, the second is species-level sorting, and the third species identification or description. Fortunately, centuries of collecting have yielded many of the specimens that are needed for large-scale species discovery. Indeed, for many invertebrate groups it is likely that museum collections contain more specimens of undescribed than described species; that is, this unsorted collection material represents vast and still underutilized source for species discovery (Lister et al. 2011; Kemp 2015; Yeates et al. 2016). The second step in species discovery is species-level sorting, which is in dire need of acceleration. Traditionally, it starts with sorting unsorted material into major taxa (e.g., order-level in insects).

This task can be accomplished by parataxonomists but may in the future be guided by robots utilizing neural networks (Valan et al. 2019). In contrast, the subsequent species-level sorting is usually time-limiting because the specimens for many invertebrate taxa have to be dissected and slide-mounted before they can be sorted to species-level by highly-skilled specialists; that is, the traditional techniques are neither rapid nor cost-effective. This impediment is likely the reason why certain taxa that are known to be abundant and species-rich are particularly poorly studied (Bickel 1999).

An alternative way to sort specimens to species-level would be with DNA sequences. This approach is particularly promising for metazoan species because most multicellular animal species can be distinguished based on cytochrome c oxidase subunit I (*cox1*) barcode sequences (Hebert et al. 2003). However, such sorting requires that every specimen is barcoded. This creates cost and scalability problems when the barcodes are obtained with Sanger sequencing (see Taylor and Harris 2012). Such sequencing is currently still the standard in many barcoding studies because the animal barcode was defined as a 658-bp long fragment of *cox1* ("Folmer region": Folmer et al. 1994), although sequences >500 bp with <1% ambiguous bases are also considered BOLD-compliant (Barcode Of Life Data System: BOLD-systems.org). The 658-bp barcode was optimized for ABI capillary sequencers but it has arguably become a burden because it is not suitable for cost-effective sequencing with Illumina platforms.

Due to these constraints, very few studies have utilized DNA barcodes to sort entire samples into putative species (but see Fagan-Jeffries et al. 2018). Instead, most studies use a mixed approach where species-level sorting is carried out based on morphology before a select few specimens per morphospecies are barcoded (e.g., Riedel et al. 2010). Unfortunately, this two-step process requires considerable amounts of skilled labor and time and does not allow for an unbiased assessment of congruence-levels between morphology and DNA barcodes.

Obtaining Barcodes With High Throughput Sequencing

Fortunately, scalability and cost-effectiveness are hallmark features of high throughput sequencing technologies. These technologies are particularly suitable for sequencing the kind of degraded DNA that is typical of museum specimens. Indeed, hybrid capture has already been optimized for use with old museum specimens (Bi et al. 2013; Guschanski et al. 2013; Blaimer et al. 2016; Hedin et al. 2018; Tsai et al. 2019) and is likely to play a major role for the integration of rare species into taxonomic and systematic projects. It will be difficult and expensive, however, to apply enrichment methods to millions of specimens because such methods require time-consuming and expensive molecular protocols (e.g., specimen-specific libraries). It is thus fortunate that for most species the initial species-level pre-sorting can be achieved using barcodes that can be obtained via "tagged amplicon sequencing" on a variety of next-generation sequencing platforms ("NGS barcodes": Wang et al. 2018; Yeo et al. 2018).

These platforms, however, come with drawbacks; viz. elevated sequencing error rates and higher cost. According to the literature, sequencing a tagged amplicon with Sequel or MinION costs USD 0.18–0.20 (Hebert et al. 2018; Srivathsan et al. 2019), while the sequencing cost with Illumina is <0.01 USD (NovaSeq 6000, 250 bp PE sequencing, assuming a demultiplexing efficiency of 50%, coverage of 100×, and a flowcell yield of 800 million; USD 6900: https://research.ncsu.edu/gsl/pricing, accessed January 2020). The quality and cost differentials are likely to be overcome in the future (e.g., Yang et al. 2018), but this would still not solve the main challenge posed by full-length barcodes; that is, problems with reliably obtaining amplicons from museum specimens with degraded DNA (e.g., Hajibabaei et al. 2006). We thus submit that it is time to use empirical evidence to determine at which length and in which position mini-barcodes are as effective for pre-sorting specimens into putative species as full-length barcodes.

Mini-Barcodes

Barcodes that are shorter than the full-length barcode are often referred to as "mini-barcodes". They are obtained with primers that amplify shorter subsets of the original barcode region and have three key advantages. Firstly, several studies comparing amplification success rates for mini- and full-length barcodes for the same DNA template (Hajibabaei et al. 2006; Meusnier et al. 2008; Hajibabaei and McKenna 2012) have confirmed that mini-barcodes amplify more readily when the DNA in the sample is degraded. This property makes mini-barcodes the preferred choice for barcoding museum specimens that had been stored under suboptimal conditions for decades. The ease of amplification is also one of the reasons why mini-barcodes are the default for metabarcoding projects that rely on environmental DNA with widely varying DNA quality [e.g., gut content, feces (Deagle et al. 2006)].

A second benefit of mini-barcodes is that they can be sequenced at low cost on short-read sequencing platforms (e.g., Illumina). The maximum amplicon lengths that such platforms can accommodate is ~450 bp including primer (using 250-bp paired-end sequencing libraries), which means that the recovery of the entire 658-barcode necessitates the amplification and sequencing of two overlapping regions (Shokralla et al. 2015b). However, given the additional time and resources required, it begs the question if there is much to be gained by investing in full-length barcodes. Full-length barcodes are often automatically assumed to improve performance but more data does not automatically yield better results. There is often a saturation point beyond which additional data have little impact while increasing consumable and labor cost. Occasionally, more data can even be detrimental. For example, fast-evolving genes or third positions in protein-encoding genes can worsen the results of phylogenetic analyses addressing the relationships between old clades. Note that this could also be a concern for barcodes because of sequence variability changes across the Folmer region of COI (Roe and Sperling 2007; Pentinsaari et al. 2016). Overall, we thus submit that one should use empirical data to determine how long barcodes should be in order to be informative.

Unfortunately, the performance of mini-barcodes remains insufficiently tested despite their ubiquitous use in metabarcoding. The existing tests suffer from lack of scale (the largest study includes 6695 barcodes for 1587 species: Meusnier et al. 2008) or taxonomic scope (usually only covering one to two family-level taxa: e.g., Hajibabaei et al. 2006; Yu and You 2010). Furthermore, the tests yielded conflicting results. Hajibabaei et al. (2006) found high congruence with the full-length barcodes when species are delimited based on mini-barcodes and Meusnier et al. (2008) find similar BLAST identification rates for mini-barcodes and full-length barcodes in their *in silico* tests. However, Yu and You (2010) conceded that mini-barcodes may have worse accuracy despite having close structural concordance with the full-length barcode. In addition, Sultana et al. (2018) concluded that the ability to identify species is compromised when the barcodes are too short (<150 bp), but it remained unclear at which length/position mini-barcodes stop performing well. Furthermore, published tests of mini-barcodes are based on comparing results obtained with mini- and full-length barcodes. All conflict is then considered evidence for the failure of mini-barcodes although the Folmer region varies in nucleotide variability (Roe and Sperling 2007) and conflict alone does not settle which result is correct. Lastly, the existing tests of mini-barcodes do not include a sufficiently large number of different mini-barcodes in order to be able to detect positional and length effects across the 658-bp barcode region.

Here, we address the lack of scale in previous tests by including 20 empirical studies covering 5500 species (ca. 30,000 barcodes) and Genbank data for 20,673 species (ca. 98,000 barcodes). We test a large number of different mini-barcodes by applying a sliding window approach to generate mini-barcodes of different lengths (100, 200, 300-bp window length and 60-bp intervals) and compare the results to the performance of nine mini-barcodes with established primers (mini-barcode length: 94–407 bp). The taxonomic scope of our study is broad and includes a wide variety of metazoans ranging from earthworms to butterflies and birds. Lastly, we do not assume that molecular operational taxonomic units (mOTUs) based on full-length barcodes are more accurate than those obtained with mini-barcodes. Instead, we assess whether mOTUs obtained with different-length barcodes have different levels of congruence with morphospecies; that is, morphology is treated as a constant and we only test whether barcode length and/or position influence the number of morphospecies that are recovered. All specimens that are mis-sorted based on morphology (e.g., due to the presence of cryptic species) are equally mis-sorted for full-length barcodes and mini-barcodes that are derived from the former via shortening; that is, misidentified specimens become noise. What we assess is whether there is an additional loss of congruence between barcodes and morphology as the barcode length shrinks or mini-barcodes are in different positions within the Folmer region of COI.

We also compare the performance of different species delimitation methods. There has been substantial interest in developing algorithms for mOTU estimation, leading to the emergence of various species delimitation algorithms over the past decade (e.g., Objective Clustering: Meier et al. 2006; BPP: Yang and Rannala 2010; jmOTU: Jones et al. 2011; ABGD: Puillandre et al. 2012; BINs: Ratnasingham and Hebert 2013; PTP: Zhang et al. 2013; etc.). For the purposes of this study, we selected three algorithms that represent distance- and tree-based methods: objective clustering, automatic barcode gap discovery (ABGD), and Poisson tree process (PTP). Objective clustering utilizes an *a priori* distance threshold to group sequences into clusters, ABGD groups sequences into clusters based on an initial prior and recursively uses incremental priors to find stable partitions, while PTP utilizes the branch lengths on the input phylogeny to delimit species units. Note that PTP is frequently applied to trees derived from barcode data (e.g., Ermakov et al. 2015; Han et al. 2016; Hollatz et al. 2016). There are numerous additional techniques for species delimitation, but most require multiple markers and thus do not scale easily to millions of specimens.

In addition to species delimitation, we also examine the utility of mini-barcodes for species identification, which is the original use of DNA barcodes (Hebert et al. 2003). Species identification with barcodes is particularly valuable for the detection of target species and community characterization in metabarcoding studies (Ficetola et al. 2008; Lim et al. 2016; Morinière et al. 2019). Most of these studies tend to involve poor-quality genetic material (i.e., gut content, fecal matter, environmental DNA) and hence it is important to know whether short markers can be reliably used to assign species names to unidentified mini-barcodes. This is here tested using “best close match”, which considers those sequences correctly identified that match conspecific barcodes within a predefined threshold (Meier et al. 2006).

Materials and Methods

Data Set Selection, Alignment, Excision of Silico Mini-Barcodes

We identified 20 recent publications (2007–2017) that cited the original barcode paper by Hebert et al. (2003) and met the following criteria: (1) consist of specimens where the barcoded specimens were pre-sorted/identified based on morphology and (2) the data set had at least 500 specimens with *cox1* barcodes >656 bp (Supplementary Table S1a available on Dryad https://doi.org/10.5061/dryad.08kprr4zk). In addition, we compiled six data sets based on sequences from GenBank for species-rich metazoan taxa: Actinopterygii, Arachnida, Coleoptera, Diptera, Hymenoptera, and Lepidoptera. The clade names were used for taxonomy searches in NCBI and all mitochondrial nucleotide sequences with a length of 657–1000 bp were downloaded. To these, we added annotated *cox1* sequences from mitochondrial genomes (mitochondrial sequences of length 10,000–20,000 bp). Subsequently, we filtered for the *cox1* gene by aligning the sequences to a reference full-length *cox1* barcode using the --add function in MAFFT (Katoh and Standley 2013). This allowed for the identification of non-target sequences that were discarded. We then removed all sequences that were shorter than 657 bp, introduced indels in the multiple sequence alignment, were not identified to species, or included ambiguous species names. Four of the data sets (Actinopterygii: 21,390 barcodes, Coleoptera: 44,403 barcodes, Diptera: 56,344 barcodes, and Lepidoptera: 198,441 barcodes) were too large for a full evaluation using all species delimitation methods. We therefore randomly selected 20,000 sequences (using "=RAND()" function in Microsoft Excel) for each of these taxa and combined them with the barcodes for Arachnida ($N = 2411$) and Hymenoptera ($N = 15{,}347$); the final set comprised 97,758 barcodes for 20,673 morphospecies (Supplementary Table S1b available on Dryad).

The barcode sequences for the 20 empirical data sets were downloaded from BOLDSystems or NCBI GenBank and aligned with MAFFT v7 (Katoh and Standley 2013) with a gap opening penalty of 5.0. Using a custom Python script, we generated three sets of mini-barcodes along a "sliding window". They were of 100-, 200-, and 300-bp lengths. The first iteration began with the first base pair of the 658-bp barcode and the shifting windows jump 60 bp at each iteration, generating ten 100-bp windows, eight 200-bp windows, and six 300-bp windows. In addition, we identified nine mini-barcodes with published primers within the *cox1* Folmer region (Fig. 1 and Supplementary Table S2 available on Dryad). These mini-barcodes have been repeatedly used in the literature for a broad range of taxa. The primers for the mini-barcodes were aligned to the homologous regions of each data set with MAFFT v7 --addfragments (Katoh and Standley 2013) in order to identify the precise position of the mini-barcodes within the full-length barcode. The mini-barcode subsets from each barcode were then identified after alignment to full-length barcodes in order to mark the start and endpoint of the amplicon. The nine mini-barcodes with published primers were assessed for the 20 empirical and the six GenBank data sets.

Species Delimitation

Mini-barcodes and full-length barcodes were clustered into putative species using three species delimitation algorithms: objective clustering (Meier et al. 2006), ABGD (Puillandre et al. 2012), and PTP (Zhang et al. 2013). For objective clustering, the mOTUs were clustered at 2–4% uncorrected *p*-distance thresholds (Srivathsan and Meier 2012) using a Python script which implements the objective clustering algorithm of Meier et al. (2006) and allows for batch processing. The *p*-distance thresholds selected (2–4%) were based on literature (Meier et al. 2006, 2016; Ratnasingham and Hebert 2013). The same data sets were also clustered with ABGD (Puillandre et al. 2012) using the default range of priors and using uncorrected *p*-distances, but the minimum slope parameter (-X) was reduced in a stepwise manner (1.5, 1.0, 0.5, 0.1) if the algorithm could not find a partition. We considered the ABGD clusters at priors $P = 0.001$, $P = 0.01$, and $P = 0.04$. The priors (P) refer to the maximum intraspecific divergence and function similarly to *p*-distance thresholds at the first iteration. In the ABGD algorithm, they are refined by recursive application. Lastly, in order to use PTP, we generated maximum likelihood (ML) trees in RAxML v.8 (Stamatakis 2014) via rapid bootstrapping (-f a) under the GTRCAT model. For the 20 empirical data sets, the best tree generated for each data set was then used for species delimitation with PTP (Zhang et al. 2013) under default parameters. For the six much larger clade-based data sets, we used mPTP (Kapli et al. 2017) with the --single parameter because the original PTP algorithm took too long to yield results. In order to reduce computational time for RAxML and PTP, haplotype representatives were used for the six GenBank data sets instead of the entire data set. The remaining barcodes with identical haplotypes were then mapped back into the mOTU clusters using their respective haplotypes.

Performance Assessment

The performance of mini-barcodes was assessed using morphospecies as an external arbiter. Species-level congruence was quantified using match ratios between molecular and morphological groups (Ahrens et al. 2016). The ratio is defined as $\frac{2\times N_{match}}{N_1+N_2}\times 100$, where N_{match} is the number of clusters identical across both mOTU delimitation methods/thresholds (N_1 and N_2). Consolidated match ratios are derived by first summing all relevant numerator and denominator values before calculating the ratio. Incongruence between morphospecies and mOTUs is usually caused by a few specimens that are assigned to the "incorrect" mOTUs. Conflict at the specimen-level can thus be quantified as the number of specimens that are in mOTUs that cause conflict with morphospecies.

In order to test whether barcode length is a significant predictor of congruence, multivariate analysis of variance (MANOVA) tests were carried out in R (R Core Team 2017) with "match ratio" (species-level congruence) as the response variable and "data set" and "mini-barcode" as categorical explanatory variables. We found that most

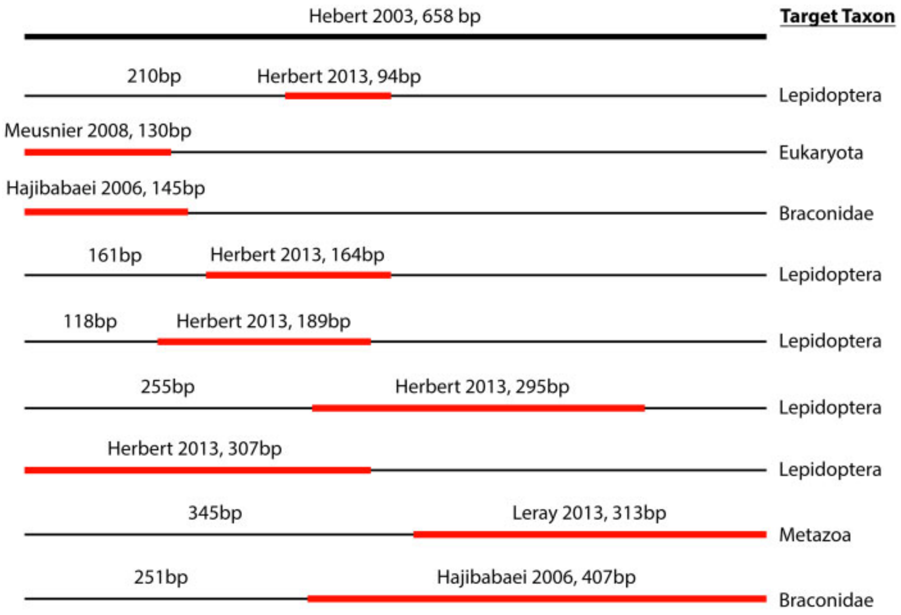


FIGURE 1. Positions of the mini-barcodes with established primers assessed in this study.

of the variance in our study was generated by the variable "data set" ($P < 0.05$ in MANOVA tests). Given that we were interested in the effect of barcode length and position, "data set" was subsequently treated as a random effect and "mini-barcode" as the explanatory variable (categorical) in a linear mixed effects model (R package *lme4*: Bates et al. 2015). The *emmeans* R package (Lenth 2018) was then used to perform pairwise *post hoc* Tukey tests between mini- and full-length barcodes in order to assess whether either barcode was performing significantly better/worse. To compare the differences in performance between objective clustering, ABGD, and PTP, analysis of variance (ANOVA) tests were performed in R. After which, pairwise Tukey tests were used to determine which species delimitation method was responsible for significant differences. Lastly, in order to explore the reasons for positional effects, the proportion of conserved sites for each mini-barcode was obtained using MEGA6 (Tamura et al. 2013).

Match ratios indicate congruence at the species level, but it is also important to determine how many specimens have been placed into congruent units. Note that species- and specimen-level congruence are only identical when all mOTUs are represented by the same number of specimens. However, specimen abundances are rarely equal across species and hence it is not sufficient to use only the match ratio for characterizing congruence between mOTUs and morphospecies. It is straightforward to determine the number of congruent specimens as follows:

(1) Congruence Class I specimens: If $A = B$ then number of congruent specimens is $\mathrm{Nc}_1 = |A|$ OR$|B|$.

Lack of congruence is caused by morphospecies that are split, lumped, or both split and lumped in the mOTUs. This means that a single mis-sorted specimen placed into a single, large-sized mOTU leads to all specimens in two mOTUs to be considered "incongruent" according to the criterion outlined above. Yet, most specimens are congruent and full congruence could be restored by re-assigning the mis-sorted specimen after re-examination. It is therefore also desirable to determine the number of specimens that require re-examination or, conversely, the number of specimens that would be congruent if one were to remove a few incongruent specimens. This number of specimens can be estimated by counting congruent specimens as follows:

(2) Congruence Class II specimens: Specimens that are in split or lumped mOTUs relative to morphospecies. Here, the largest subset of congruently placed specimens can be determined as follows. If $A_1 \cup A_2 \cup \ldots \cup A_x = B : Nc_2 = \max(|A_1|, |A_2| \ldots |A_x|)$

(3) Congruence Class III specimens: This covers specimens in sets of clusters that are both split and lumped relative to morphospecies. Here, only those specimens are considered potentially congruent that (1) are in one mOTU and one morphospecies and (2) combined exceed the number of the other specimens in the set of clusters. In detail, if $A_1 \cup A_2 \cup \ldots \cup A_x = B_1 \cup B_2 \cup \ldots \cup B_y : Nc_3 = \max(|A_1 \cap B_1|, |A_2 \cap B_1| \ldots |A_x \cap B_y|)$ only if $\max(|A_1 \cap B_1|, |A_2 \cap B_1| \ldots |A_x \cap B_y|) > \frac{1}{2}(|A_1 \cup A_2 \ldots A_x|)$.

Note that these are likely (over)estimates of incongruence because we did not re-cluster specimens after the removal of the specimens that were causing incongruence in the original sets of clusters.

Species Identification

Using the full-length and mini-barcode sets corresponding to published primers, we assessed whether the barcodes were yielding conspecific matches according to "best close match" implemented in the SpeciesIdentifier (TaxonDNA: Meier et al. 2006). "Best close match" examines one barcode at a time which is considered unidentified by ignoring its species-label. It is then determined whether the best-matching reference sequence in the remaining data set is conspecific and within a user-defined distance threshold. The distance threshold used for all assessments in our study was 2%, which corresponds to the best match ratio performance results for objective clustering in this study. Sequences with conspecific matches within 2% are considered correctly identified, while sequences that match allo-specific sequences within 2% are considered incorrectly identified. Sequences without matches within 2% are considered unidentified and sequences that have both con- and allo-specific matches within the threshold are considered to have an ambiguous identification. Mixed effects models were again used to assess the performance of barcodes of different length. Similarly, pairwise Tukey tests were used to test for statistical significance with the proportion of correct matches as the response variable in order to determine whether the full-length barcode performed significantly better than the mini-barcodes.

Amplification Success Rates For 313-bp and 658-bp Amplicons

We tested amplification success rates for 47–48 specimens (~1 mm) from six Malaise trap samples of different age (2012 and 2018). The same genomic DNA was used for each specimen for the amplification of the full-length barcode and a mini-barcode of 313-bp length in two separate PCRs using different tagged primers: HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) and LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) (658-bp; Folmer et al. 1994), mlCOIintF (5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′), and jgHCO2198 (5′TAIACYTCIGGRTGICCRAARAAYCA-3′) (313-bp; Leray et al. 2013). The PCR reagents used were 5.0 μL Taq MasterMix (CW0682; CWBio, Beijing, China), 1.0 μL RNase-free water, 0.5 μL bovine serum albumin (1.0 mM), 0.5 μL $MgCl_2$, 1.0 μL of each primer, and 1.0 μL of genomic DNA extract. The PCRs were run at 95°C for 5 min, with 35 cycles of 94°C for 1 min, 45°C for 2 min and 72°C for 1 min, and a final extension at 72°C for 5 min. PCR amplification success was assessed using a 1% agarose gel, with the electrophoresis run at 90 V for 30 min. Band presence was tabulated and χ^2 performance testing was performed in R (R Core Team 2017).

Results

Evaluation of Mini-Barcode Congruence With Morphology

For species delimitation with objective clustering, we found that the 2% *p*-distance threshold maximized congruence across the data sets. It was hence used as the upper-bound estimator for assessing species- and specimen-level congruence (see Supplementary materials available on Dryad for corresponding results for the 3% and 4% *p*-distances). For ABGD, average congruence was maximized for the prior $P = 0.001$ and this prior was used in the main analysis (see Supplementary material available on Dryad for results under $P = 0.01$ and $P = 0.04$). For the third species-delimitation method, PTP, no *a priori* parameter choices were needed. Overall, the consolidated match ratio values range from 68% to 74% for the three species delimitation methods (Fig.), with the 20 empirical data sets generally performing better than the six clade-based data sets (empirical/clade-based: OC at 2%: 81/72%; ABGD $P = 0.001$: 76/69%; PTP: 76/66%). The match ratios for other parameters tested with objective clustering and ABGD were similar (61–74%: Supplementary Fig. S6 available on Dryad).

The MANOVA tests performed on all treatments (species delimitation method and distance threshold/prior) indicated that the test variable "data set" was responsible for much of the observed variance in our measure of congruence ("match ratio"). The choice of mini-barcode or algorithm for generating mOTUs was of secondary importance (Supplementary Table S3 available on Dryad). After accounting for "data set", we find that only mini-barcodes <200 bp perform significantly worse than full-length barcodes (Fig. 2). This is evident from the large number of significant differences ($P < 0.05$ and $P < 0.001$) in pairwise *post hoc* Tukey tests applied to 100-bp mini- and 657-bp full-length barcodes. Only short barcodes (<100 bp) have a mean performance that is worse (<0 match ratio deviation) than the full-length barcode. Conversely, for mini-barcodes >200 bp, congruence with morphospecies does not differ significantly and is occasionally superior to what is observed for the full-length barcode.

The full-length barcode yields the highest congruence values in only 49 of 182 tests carried out across all data sets (20 empirical and 6 clade-based), species delimitation methods and clustering parameters (Fig. and Supplementary Fig. S6 available on Dryad). In eight of these cases, it is a tie, and in an additional 19 cases, some mini-barcodes are within 0.5% of the results obtained with the full-length barcode. However, overall there are more cases where minibarcodes outperform full-length barcodes (133 including 8 ties and 19 cases within 0.5%). This illustrates that there is no significant difference between the 200-bp and 300-bp mini-barcodes and the full-length barcode when objective clustering or PTP are used to estimate mOTUs, but the variance across data sets declines as the mini-barcode increases in length (Fig. 2). The results obtained for *in silico* mini-barcodes are broadly consistent with the performance of

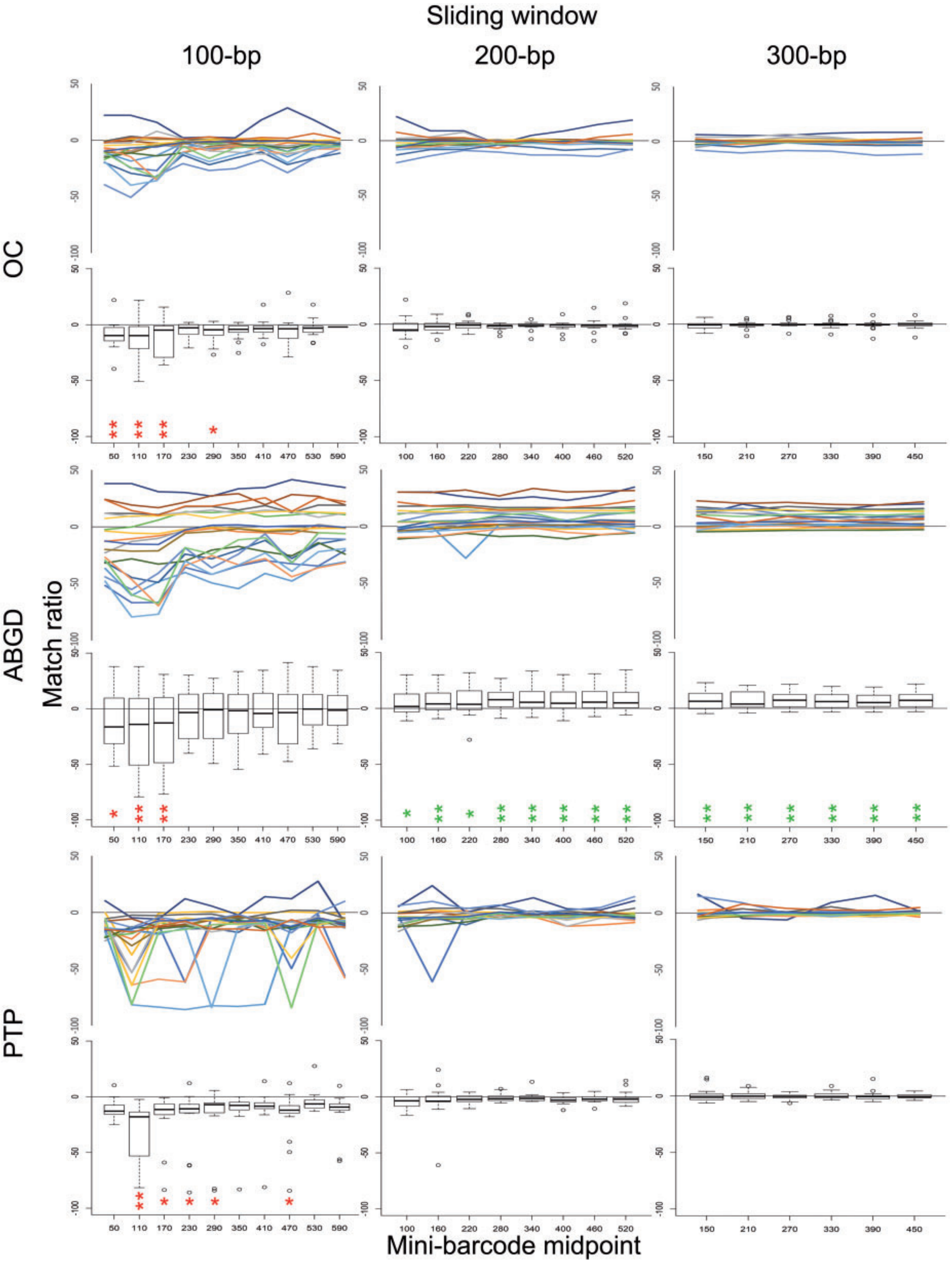


FIGURE 2. Performance of mini-barcodes along a sliding window (100-, 200, and 300-bp). Mini-barcode position is indicated on the x-axis and congruence with morphology on the y-axis. mOTUs were obtained with objective clustering (2%), ABGD $P=0.001$ prior), and PTP. Each line represents one data set, while the boxplots summarize the values across data sets. Significant deviations from the results obtained with full-length barcodes are indicated with color-coded asterisks (*$P < 0.05$; **$P < 0.001$; red = poorer and green = higher congruence with morphology).

mini-barcodes corresponding to published primers: the mini-barcodes of 94-bp, 130-bp, and 145-bp lengths tend to perform worse than the longer mini-barcodes (Fig.). Congruence at the species level is similar to congruence at the specimen level (Table 1 and Supplementary Table S8 available on Dryad) with some exceptions such as performance improvements of short mini-barcodes, for example, for the "French Guiana earthworms" when grouped with objective clustering.

The results obtained for the 20 empirical data sets are similar to what is found for the taxon-specific data sets consisting of sequences from GenBank. There are small differences in match ratios for barcodes >200 bp and only very short barcodes perform poorly. In many cases, the full-length barcode does not yield the best performance. An exception is Coleoptera where ABGD behaves erratically (Fig.): short barcodes outperform long barcodes under the lowest prior (P=0.001), but this trend reverses for higher priors.

With regard to specimen-based congruence, we focused on the mini-barcodes with published primers that are >200 bp. For the 20 empirical data sets, approximately three quarters of all specimens are in the "Congruence Class I" (Table 1 and Supplementary Table S8 available on Dryad); that is, their placement is congruent between mOTUs and morphospecies (Average/Median: OC at 2%: 77/77%; ABGD P=0.001: 74/74%; PTP: 77/77%). The remaining specimens are placed in mOTUs that are split, lumped, or split and lumped. The number of specimens that are responsible for splitting and lumping are classified as Congruence Class II and III specimens (see Materials and Methods section). Overall, fewer than 10% of the specimens fall into these categories (Table 1: see Class II specimens across species delimitation methods); that is, a fairly small number of specimens have to be studied when addressing conflict between morphospecies and mOTUs. For the six clade-based data sets, there is generally lower specimen congruence (Class I Average/Median: OC at 2%: 57/57%; ABGD P= 0.001: 53/54%; PTP: 57/57%), although this improves to ca. 75% at Class II and ca. 80% at Class III. Note that a single mis-identified *Drosophila melanogaster* could result in all correctly identified specimens of this species to be considered "incongruent" because they are no longer member of a congruent mOTU.

Species Delimitation Method

When the performance of the three different clustering methods was compared, significant differences ($P <$ 0.05 in ANOVA test) were found only for 100-bp mini-barcodes (Fig. 4). Here, pairwise *post hoc* Tukey tests find that objective clustering performs significantly better than the other delimitation methods ($P <$ 0.001), while ABGD and PTP do not differ significantly (P= 0.88) but behave erratically for short mini-barcodes (Fig. 2).

Positional Effects

Mini-barcodes situated at the 5′ end of the full-length barcode perform somewhat worse than those situated at the middle or at the 3′ end (Fig. 2). For example, the 100-bp mini-barcodes at the 5′ end perform poorly for objective clustering (mini-barcode midpoints at 50, 110, and 170 bp), ABGD (mini-barcode midpoints at 110 and 170 bp), and PTP (mini-barcode midpoint at 110 bp). This effect is, however, only statistically significant when the mini-barcodes are very short (100 bp). This positional effect is observed across all species delimitation techniques. Note that the 5′ end of the full-length barcode appears to contain a large proportion of conserved sites, particularly around the 170-bp and 230-bp midpoint of the 100-bp mini-barcode (Fig. 5).

Species Identification Performance

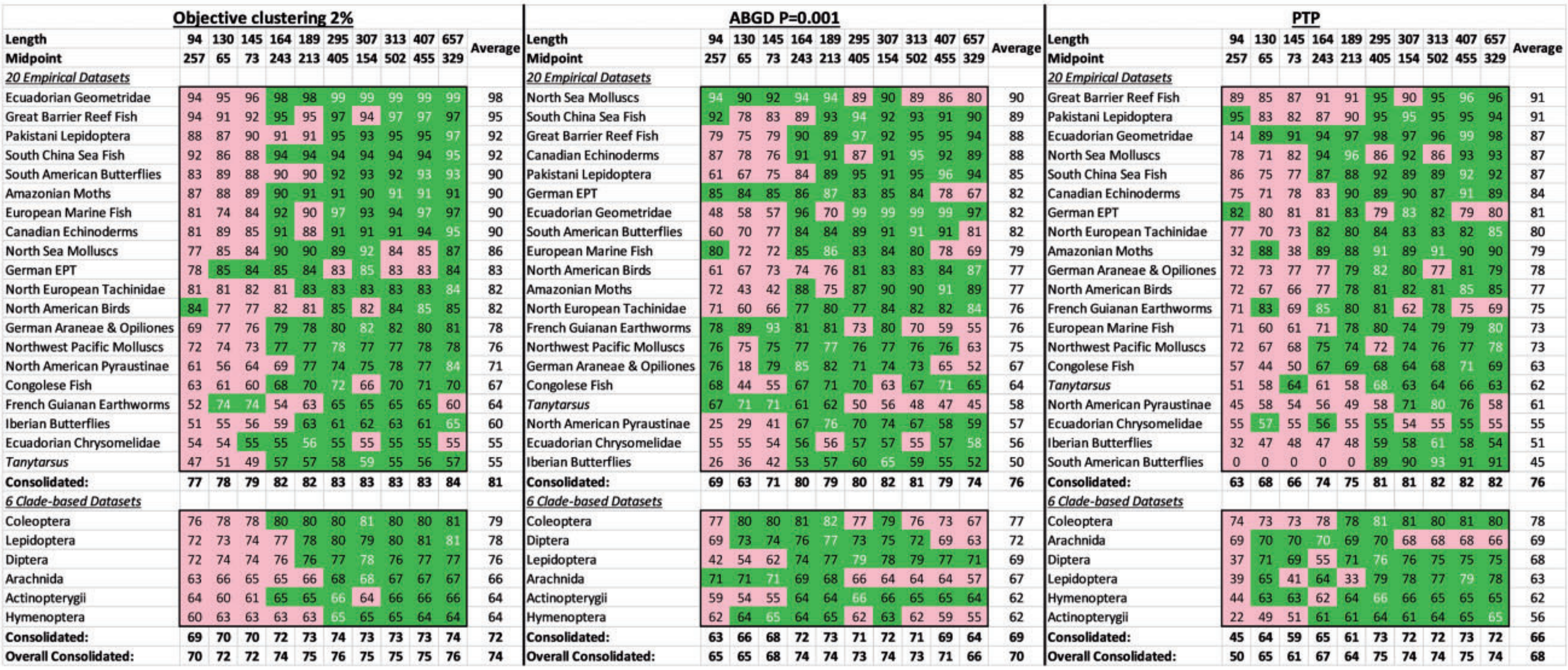
When comparing the suitability of full-length and mini-barcodes for identification purposes, we find that full-length barcodes frequently have the highest number of correct matches (Table 2), but the differences are only statistically significant for short mini-barcodes <200 bp: 94 bp: $P \leqslant$ 0.0001, 130 bp: $P \leqslant$ 0.0001, 145 bp: $P \leqslant$ 0.0001, and 164 bp: P= 0.0243. All the remaining mini-barcodes (189–407 bp) have non-significant differences (P=0.0691−0.9996). Note that this is not due to scale because we are using fairly large data sets here. However, for many data sets (e.g., Northwest Pacific Molluscs, North American Birds, etc.), the proportion of ambiguous matches is higher for very short mini-barcodes (94–164 bp).

Amplification Success Rates

The 313-bp mini-barcode amplified significantly better than the full-length barcode (472/570: 82.8% vs. 361/570: 63.3%; χ^2 test: $P \leqslant$ 0.001) (Supplementary Fig. S9 available on Dryad). Overall, the performance differences were greater for samples with overall low amplification success rates (but see sample 4822).

Discussion

Accelerating species discovery and description are arguably some of the foremost challenges for the systematics of the 21st century because biodiversity is in steep decline, while systematists are still grappling with establishing the size and distribution of the biodiversity that needs protection. Specimens for many undescribed species are already in the world's natural history museums, but the samples need to be sorted to species-level before they become available for species identification/description and can be used for large-scale analyses of biodiversity patterns. Pre-sorting specimens with DNA barcodes is a potentially promising solution because it is scalable, can be applied to millions



Objective clustering 2%

Length	94	130	145	164	189	295	307	313	407	657	Average
Midpoint	**257**	**65**	**73**	**243**	**213**	**405**	**154**	**502**	**455**	**329**	
20 Empirical Datasets											
Ecuadorian Geometridae	94	95	96	98	98	99	99	99	99	99	98
Great Barrier Reef Fish	94	91	92	95	95	97	94	97	97	97	95
Pakistani Lepidoptera	88	87	90	91	91	95	93	95	95	97	92
South China Sea Fish	92	86	88	94	94	94	94	94	94	95	92
South American Butterflies	83	89	88	90	90	92	93	92	93	93	90
Amazonian Moths	87	88	89	90	91	91	90	91	91	91	90
European Marine Fish	81	74	84	92	90	97	93	94	97	97	90
Canadian Echinoderms	81	89	85	91	88	91	91	91	94	95	90
North Sea Molluscs	77	85	84	90	90	89	92	84	85	87	86
German EPT	78	85	84	85	84	83	85	83	83	84	83
North European Tachinidae	81	81	82	81	83	83	83	83	83	84	82
North American Birds	84	77	77	82	81	85	82	84	85	85	82
German Araneae & Opiliones	69	77	76	79	78	80	82	82	80	81	78
Northwest Pacific Molluscs	72	74	73	77	77	78	77	77	78	78	76
North American Pyraustinae	61	56	64	69	77	74	75	78	77	84	71
Congolese Fish	63	61	60	68	70	72	66	70	71	70	67
French Guianan Earthworms	52	74	74	54	63	65	65	65	65	60	64
Iberian Butterflies	51	55	56	59	63	61	62	63	61	65	60
Ecuadorian Chrysomelidae	54	54	55	55	56	55	55	55	55	55	55
Tanytarsus	47	51	49	57	57	58	59	55	56	57	55
Consolidated:	**77**	**78**	**79**	**82**	**82**	**83**	**83**	**83**	**83**	**84**	**81**
6 Clade-based Datasets											
Coleoptera	76	78	78	80	80	80	81	80	80	81	79
Lepidoptera	72	73	74	77	78	80	79	80	81	81	78
Diptera	72	74	74	76	76	77	78	76	77	77	76
Arachnida	63	66	65	65	66	68	68	67	67	67	66
Actinopterygii	64	60	61	65	65	66	64	66	66	66	64
Hymenoptera	60	63	63	63	63	65	65	65	64	64	64
Consolidated:	**69**	**70**	**70**	**72**	**73**	**74**	**73**	**73**	**73**	**74**	**72**
Overall Consolidated:	**70**	**72**	**72**	**74**	**75**	**76**	**75**	**75**	**75**	**76**	**74**

ABGD P=0.001

Length	94	130	145	164	189	295	307	313	407	657	Average
Midpoint	**257**	**65**	**73**	**243**	**213**	**405**	**154**	**502**	**455**	**329**	
20 Empirical Datasets											
North Sea Molluscs	94	90	92	94	94	89	90	89	86	80	90
South China Sea Fish	92	78	83	89	93	94	92	93	91	90	89
Great Barrier Reef Fish	79	75	79	90	89	97	92	95	95	94	88
Canadian Echinoderms	87	78	76	91	91	87	91	95	92	89	88
Pakistani Lepidoptera	61	67	75	84	89	95	91	95	96	94	85
German EPT	85	84	85	86	87	83	85	84	78	67	82
Ecuadorian Geometridae	48	58	57	96	70	99	99	99	99	97	82
South American Butterflies	60	70	77	84	84	89	91	91	91	81	82
European Marine Fish	80	72	72	85	86	83	84	80	78	69	79
North American Birds	61	67	73	74	76	81	83	83	84	87	77
Amazonian Moths	72	43	42	88	75	87	90	90	91	89	77
North European Tachinidae	71	60	66	77	80	77	84	82	82	84	76
French Guianan Earthworms	78	89	93	81	81	73	80	70	59	55	76
Northwest Pacific Molluscs	76	75	75	77	77	76	77	76	76	63	75
German Araneae & Opiliones	76	18	79	85	82	71	74	73	65	52	67
Congolese Fish	68	44	55	67	71	70	63	67	71	65	64
Tanytarsus	67	71	71	61	62	50	56	48	47	45	58
North American Pyraustinae	25	29	41	67	76	70	74	67	58	59	57
Ecuadorian Chrysomelidae	55	55	54	56	56	57	57	55	57	58	56
Iberian Butterflies	26	36	42	53	57	60	65	59	55	52	50
Consolidated:	**69**	**63**	**71**	**80**	**79**	**80**	**82**	**81**	**79**	**74**	**76**
6 Clade-based Datasets											
Coleoptera	77	80	80	81	82	77	79	76	73	67	77
Diptera	69	73	74	76	77	73	75	72	69	63	72
Lepidoptera	42	54	62	74	77	79	78	79	77	71	69
Arachnida	71	71	71	69	68	66	64	64	64	57	67
Actinopterygii	59	54	55	64	64	66	66	65	65	64	62
Hymenoptera	62	64	65	64	65	62	63	62	59	55	62
Consolidated:	**63**	**66**	**68**	**72**	**73**	**71**	**72**	**71**	**69**	**64**	**69**
Overall Consolidated:	**65**	**65**	**68**	**74**	**74**	**73**	**74**	**73**	**71**	**66**	**70**

PTP

Length	94	130	145	164	189	295	307	313	407	657	Average
Midpoint	**257**	**65**	**73**	**243**	**213**	**405**	**154**	**502**	**455**	**329**	
20 Empirical Datasets											
Great Barrier Reef Fish	89	85	87	91	91	95	90	95	96	96	91
Pakistani Lepidoptera	95	83	82	87	90	95	95	95	95	94	91
Ecuadorian Geometridae	14	89	91	94	97	98	97	96	99	98	87
North Sea Molluscs	78	71	82	94	96	86	92	86	93	93	87
South China Sea Fish	86	75	77	87	88	92	89	89	92	92	87
Canadian Echinoderms	75	71	78	83	90	89	90	87	91	89	84
German EPT	82	80	81	81	83	79	83	82	79	80	81
North European Tachinidae	77	70	73	82	80	84	83	83	82	85	80
Amazonian Moths	32	88	38	89	88	91	89	91	90	90	79
German Araneae & Opiliones	72	73	77	77	79	82	80	77	81	79	78
North American Birds	72	67	66	77	78	81	82	81	85	85	77
French Guianan Earthworms	71	83	69	85	80	81	62	78	75	69	75
European Marine Fish	71	60	61	71	78	80	74	79	79	80	73
Northwest Pacific Molluscs	72	67	68	75	74	72	74	76	77	78	73
Congolese Fish	57	44	50	67	69	68	64	68	71	69	63
Tanytarsus	51	58	64	61	58	68	63	64	66	63	62
North American Pyraustinae	45	58	54	56	49	58	71	80	76	58	61
Ecuadorian Chrysomelidae	55	57	55	56	55	55	54	55	55	55	55
Iberian Butterflies	32	47	48	47	48	59	58	61	58	54	51
South American Butterflies	0	0	0	0	0	89	90	93	91	91	45
Consolidated:	**63**	**68**	**66**	**74**	**75**	**81**	**81**	**82**	**82**	**82**	**76**
6 Clade-based Datasets											
Coleoptera	74	73	73	78	78	81	81	80	81	80	78
Arachnida	69	70	70	70	69	70	68	68	68	66	69
Diptera	37	71	69	55	71	76	76	75	75	75	68
Lepidoptera	39	65	41	64	33	79	78	77	79	78	63
Hymenoptera	44	63	63	62	64	66	66	65	65	65	62
Actinopterygii	22	49	51	61	61	64	61	64	65	65	56
Consolidated:	**45**	**64**	**59**	**65**	**61**	**73**	**72**	**72**	**73**	**72**	**66**
Overall Consolidated:	**50**	**65**	**61**	**67**	**64**	**75**	**74**	**74**	**75**	**74**	**68**

FIGURE 3. Match ratios across three different species delimitation methods. Mini-barcodes (columns) are sorted by primer length, while the 20 empirical data sets (rows) are sorted by average match ratio. The six clade-specific data sets from GenBank are shown below. Match ratios above average = green; match ratios below average = pink; highest match ratio = white text; note that only integers are shown, but comparisons were made at 2 decimal places.

TABLE 1. Proportion of specimens congruent between morphospecies and mOTUs (20 empirical data sets/6 clade-based data sets) under the three stringency criteria

Length	295	307	313	407	657	Average	Median
Midpoint	405	154	502	455	329		
Objective clustering, 2% *p*-distance							
Class	77/57% (7475/41,367)	76/56% (7511/42,052)	77/57% (7275/40,857)	77/57% (7246/40,849)	77/58% (7118/40,327)	77/57% (7325/41,090)	77/57% (7372/40,941)
Class II	90/75% (3283/23,846)	90/75% (3333/24,794)	90/75% (3131/23,554)	90/76% (3078/23,304)	90/76% (3006/23,349)	90/75% (3166/23,769)	90/76% (3112/23,469)
Class III	91/82% (3021/17,875)	91/81% (2954/18,981)	91/82% (2822/17,666)	91/82% (2813/17,511)	91/82% (2759/17,509)	91/82% (2874/17,908)	91/82% (2862/17,745)
ABGD, $P=0.00$							
Class	75/55% (7916/42,576)	76/55% (7685/42,403)	75/55% (8169/42,787)	73/53% (8583/45,131)	69/47% (9833/49,848)	74/53% (8437/44,549)	74/54% (8126/43,386)
Class II	89/75% (3305/24,555)	89/75% (3442/24,541)	89/75% (3336/24,267)	89/74% (3526/25,153)	88/72% (3901/26,691)	89/74% (3502/25,041)	89/74% (3382/24,625)
Class III	91/81% (2981/18,303)	91/81% (2906/18,509)	90/81% (2984/18,352)	90/80% (3041/18,978)	89/78% (3482/20,860)	90/80% (3079/19,000)	91/81% (2927/18,494)
PTP							
Class	76/57% (7643/40,692)	76/56% (7744/42,608)	77/57% (7369/41,588)	78/57% (7130/41,056)	78/58% (7207/40,234)	77/57% (7419/41,236)	77/57% (7318/40,839)
Class II	89/75% (3322/23,946)	89/75% (3549/24,598)	90/75% (3230/23,857)	90/76% (3109/23,583)	90/76% (3127/22,912)	90/75% (3267/23,779)	90/76% (3198/23,659)
Class III	90/81% (3091/18,208)	90/81% (3334/19,189)	91/82% (2926/18,179)	91/81% (2813/18,968)	91/82% (2859/17,317)	91/81% (3005/18,372)	91/81% (2937/18,193)

Notes: Values in brackets represent an upper bound estimate of number of specimens causing conflict.

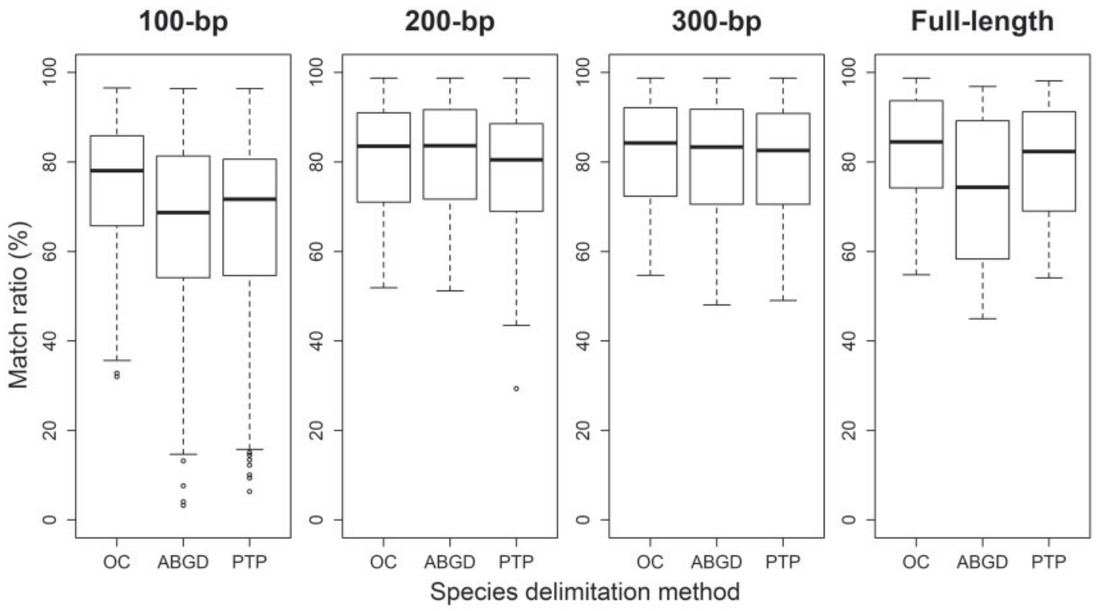


FIGURE 4. Performance of species delimitation methods for full-length and mini-barcodes generated by "sliding windows" (100-, 200-, and 300-bp).

of specimens, and much of the specimen handling can be automated. However, in order for this approach to be viable, a sufficiently large proportion of the presorted units need to accurately reflect species boundaries. Furthermore, the methods should be suitable for processing specimens that only yield degraded DNA. Mini-barcodes appear attractive on technical grounds because obtaining short amplicons is easier when the DNA template is degraded (see Hajibabaei et al. 2006; Meusnier et al. 2008; Hajibabaei and McKenna 2012).

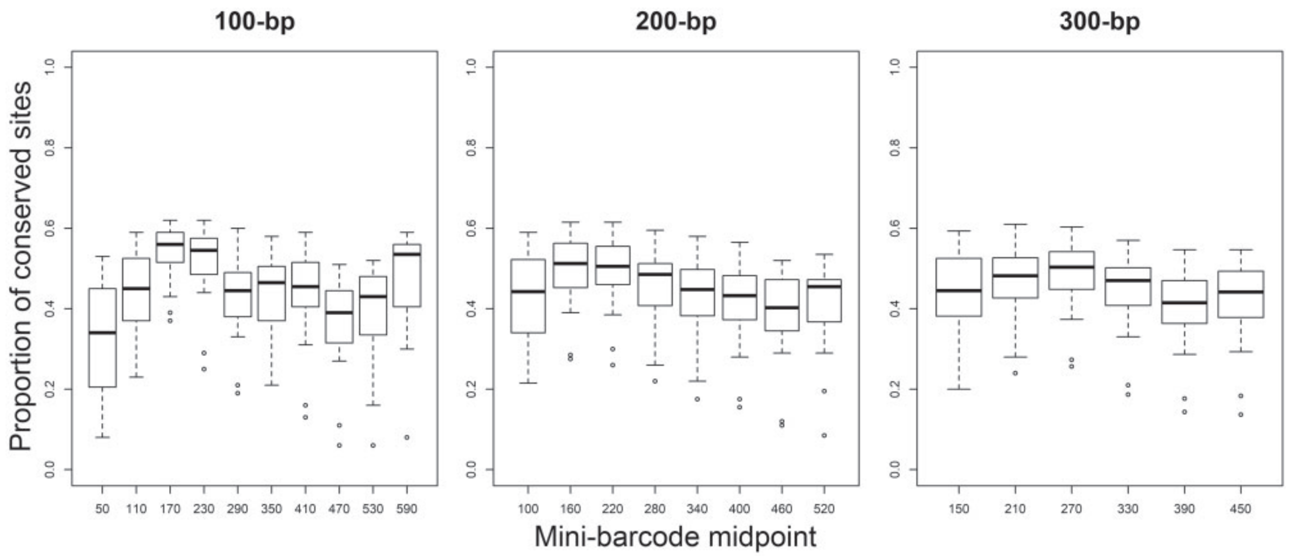


FIGURE 5. Proportion of conserved sites along the full-length barcode (sliding windows of 100-, 200, and 300-bp).

TABLE 2. Proportion of correct/incorrect/ambiguous/unidentified specimens in the "best close match" analysis

	Published primer									
Data set	94	130	145	164	189	295	307	313	407	657
Great Barrier Reef Fish	85/1/0/15	83/1/3/13	83/1/2/14	85/0/0/14	86/1/0/13	86/0/0/14	85/1/0/14	86/0/0/14	86/0/0/14	86/0/0/14
South China Sea Fish	93/0/1/6	91/1/3/6	91/1/3/6	93/0/1/6	93/0/1/6	94/0/0/6	93/0/1/6	93/1/1/6	94/1/0/6	94/0/0/6
North Sea Molluscs	90/0/0/10	91/0/0/9	92/0/0/8	92/0/0/8	92/0/0/8	92/0/0/8	92/0/0/8	91/0/0/9	91/0/0/9	92/0/0/8
Canadian Echinoderms	95/0/0/5	95/0/0/4	95/0/0/5	96/0/0/4	96/0/0/4	95/0/0/5	96/0/0/4	95/0/0/5	96/0/0/4	96/0/0/4
Ecuadorian Geometridae	84/0/0/15	85/1/0/13	85/0/0/14	85/0/0/14	85/0/0/14	86/0/0/14	86/0/0/14	86/0/0/14	86/0/0/14	86/0/0/14
German Araneae and Opiliones	88/0/5/7	89/0/5/5	89/0/5/6	90/1/4/5	90/0/4/5	92/0/2/6	91/1/3/5	92/0/2/5	92/1/2/5	93/1/2/5
European Marine Fish	92/0/7/1	91/0/7/1	92/0/6/2	96/0/3/1	96/0/3/1	97/0/1/1	97/0/1/1	96/0/2/1	97/0/1/1	98/0/1/1
South American Butterflies	89/0/4/7	91/1/2/6	92/0/1/6	92/0/1/6	93/0/0/6	93/0/0/6	94/0/0/6	94/0/0/6	94/0/0/6	94/0/0/6
German EPT	92/0/1/7	93/0/1/5	94/0/1/6	94/0/0/6	94/0/0/6	93/0/0/6	94/0/0/5	94/0/0/6	94/0/0/6	94/0/0/6
Northwest Pacific Molluscs	69/2/15/14	75/2/10/13	74/2/10/13	73/2/12/12	73/2/12/12	77/2/8/12	77/2/9/12	76/3/9/12	77/3/8/12	78/4/6/12
Amazonian Moths	62/2/1/36	63/3/1/34	63/3/1/34	62/2/1/35	63/2/1/35	63/1/1/35	63/2/1/34	63/1/1/35	63/1/1/35	63/2/1/34
North American Birds	77/1/11/11	72/2/15/10	73/2/15/11	81/2/7/10	80/1/9/10	83/2/5/11	81/2/6/11	84/2/3/10	85/2/3/10	85/2/2/11
French Guianan Earthworm	96/0/0/4	97/0/0/3	97/0/0/3	96/0/0/4	96/0/0/4	97/0/0/3	97/0/0/3	97/0/0/3	97/0/0/3	96/0/0/4
Pakistani Lepidopter	88/1/3/8	90/1/1/8	90/1/1/8	91/1/1/8	91/1/0/8	91/0/0/8	91/0/0/8	91/0/0/8	91/0/0/8	91/0/0/8
Tanytarsus	81/1/5/12	83/2/5/11	82/2/5/12	86/2/2/11	85/2/3/10	83/2/3/12	86/2/3/9	85/2/2/10	86/2/1/11	87/2/1/11
North European Tachinida	64/2/14/19	65/3/16/17	65/3/15/17	66/3/13/18	65/3/15/17	67/3/11/18	68/3/12/17	68/3/10/18	69/3/10/18	71/4/8/17
Congolese Fish	80/1/10/9	78/1/11/9	79/1/11/10	84/2/6/7	86/2/5/7	85/2/5/8	86/2/4/8	86/3/4/7	86/3/4/7	88/3/2/7
North American Pyraustinae	93/1/4/3	94/1/4/1	96/1/2/1	96/1/1/2	97/0/0/2	97/0/0/3	98/1/0/2	97/0/0/3	97/0/0/2	98/2/0/2
Ecuadorian Chrysomelidae	74/0/1/25	76/0/1/23	76/0/1/23	76/0/1/23	76/0/1/24	75/0/1/24	76/0/1/23	75/0/1/24	75/0/1/24	75/0/1/24
Iberian Butterflie	77/0/22/1	85/0/15/0	86/0/13/0	87/0/12/0	89/0/10/0	93/0/6/0	94/1/5/0	94/0/5/0	96/0/4/0	97/1/3/0

This pattern is also confirmed in our study when we compared the amplification success rates for a mini-barcode (313 bp) and the full-length barcode for the same templates (Supplementary Fig. S9 available on Dryad). In addition, sequencing short amplicons (<400 bp) is cheap because Illumina platforms can be used. Assuming 100× coverage and a 50% demultiplexing efficiency, the cost of sequencing a single barcode on a NovaSeq 6000 is <1 cent (see Introduction section).

However, the use of mini-barcodes for species-level pre-sorting would nevertheless have to be discouraged if full-length barcodes were to significantly outperform mini-barcodes with regard to the accuracy of sorting specimens into species. This is what we tested here and we find that mini-barcodes of moderate length (>200 bp) perform as well as full-length barcodes.

The Main Source of Variance of Congruence: Data Sets

Overall, we here find that the average congruence between mOTUs and morphospecies is 80% for all barcodes >200 bp (median: 83%) in the 20 empirical data sets, with the median being higher (83%) because of a few outlier data sets with low congruence <65% (OC at 2%; ABGD P=0.001, PTP). Similar variance is also observed for the six clade-specific GenBank data sets. Both types of data sets were tested because GenBank data are less likely to be affected by the idiosyncrasies of individual studies while potentially being more problematic because of misidentified sequences (Mioduchowska et al. 2018). We overall find that the results are nevertheless similar (Fig. and Supplementary Fig. S6 available on Dryad) although the average congruence between mOTUs and morphospecies is somewhat lower for the GenBank data sets (mean = 72%, median = 71% for all barcodes >200 bp across all three species delimitation methods and parameters). Full-length barcodes only have the highest congruence level in 13 of the 42 treatments, but in 10 of those, the second highest congruence level for a mini-barcode was only <0.5% lower. Mini-barcodes <200 bp perform poorly with the possible exception of those for Coleoptera, where mini-barcodes <200 bp perform well under two priors applied to ABGD (Fig. and Supplementary Fig. S6 available on Dryad: P=0.001, P=.01).

Most studies assessing congruence levels between barcodes and morphology focus on species-level congruence. However, specimen-level congruence is equally important because the basic units in a museum collection or ecological survey are specimens. The correct placement of specimens into species is thus important for systematists and biodiversity researchers alike, given that the former would like to see most of the specimens in a collection correctly sorted and the latter often needs abundance and biomass information with species-level resolution. We find that for the 20 empirical data sets, 74–77% (median) of the ca. 30,000 specimens are assigned to species that are supported by molecular and morphological data even under the strictest conditions (Table 1). Overall, this is a very high proportion compared with the species-level congruence obtained with species-level sorting by parataxonomists (Krell 2004).

The remaining ca. 25% of specimens are placed in mOTUs whose boundaries do not agree with morphospecies. One may initially consider this an unacceptably high proportion, but it is important to keep in mind that the misplacement of one specimen (e.g., due to a misidentification or contamination of a PCR product) will render two mOTUs incongruent; that is, all specimens in these mOTUs will be considered incongruent and contribute to the 25% of “incongruently” placed specimens. Arguably, one may instead want to investigate how many specimens are causing the conflict. These are the specimens that should be targeted for re-investigation in reconciliation studies using additional data. The proportion across the 20 data sets in our study is fairly low and ranges from 9% to 11% (median) depending on which mOTU delimitation technique is used.

The six GenBank data sets, however, have notably lower congruence-levels for specimens (Class I median: 54–57%), with the Actinopterygii (47–48% median) and Hymenoptera (45–47% median) having the poorest performance (Supplementary Table S8 available on Dryad). The overall lower congruence could be caused by the misidentification of a few specimens belonging to large mOTUs. This is supported by the greater difference between Class I and Class III for the clade-based data sets as compared with the empirical ones. Identifications from the empirical data sets are more likely to be standardized within the study and consequently have fewer potential conflicts caused by differing taxon concepts associated with the same name (Franz 2005; Meier 2017).

Conflict between mOTUs and morphospecies can be caused by technical error or biology. A typical technical factor would be accidental misplacement of specimens due to lab contamination or error during morphospecies sorting. Indeed, the literature is replete with cases where mOTUs that were initially in conflict with morphospecies became congruent once the study of additional morphological characters led to the revision of morphospecies boundaries (e.g., Smith et al. 2008; Tan et al. 2010; Baldwin et al. 2011; Ang et al. 2017). But there are also numerous biological reasons why one should not expect perfect congruence between mOTUs and species. Lineage sorting, fast speciation, large amounts of intraspecific variability and introgression are known to negatively affect the accuracy of DNA barcodes (Will and Rubinoff 2004; Rubinoff et al. 2006; Meier 2008). It is thus somewhat heartening that regardless of these issues, the levels of congruence between morphospecies and DNA sequences are often quite high in animals (Ball et al. 2005; Cywinska et al. 2006; Renaud et al. 2012; Landi et al. 2014; Wang et al. 2018). This implies that pre-sorting specimens to species-level units based on barcodes is worth pursuing for many metazoan clades. This realization led to the proposal of the “reverse workflow" in Wang et al. (2018) who tested pre-sorting based on sequences for 4,000 ant specimens. They found that after reconciliation, 86 of the 89 mOTUs were congruent with morphospecies.

High levels of congruence are, however, not a universal observation across all of life. There are groups with widespread barcode sharing between species. Within Metazoa this is known for taxa such as Anthozoa (Huang et al. 2008) and is likely to be the default outside of Metazoa (e.g., Chase and Fay 2009; Hollingsworth et al. 2011). Indeed, congruence levels between mOTUs and morphospecies also vary widely in the 20 empirical and 6 Genbank data sets studied here (Fig.). Eighteen of the 26 data sets have congruence levels >75% and are arguably doing well. With regard to the remaining eight, there are four where the low levels may be due to the fact that they cover taxa or faunas that are poorly known which may have caused problems with sorting based on morphology (French Guiana Earthworms, Ecuadorian Chrysomelidae, chironomid midges in the genus *Tanytarsus*). For a 5th data set, "Congolese fishes", the authors also mention identification problems due to the lack of identification tools (Decru et al. 2016). However, none of the these problems are likely to plague a data set like "Iberian butterflies", where the authors consider it likely that introgression and cryptic species are responsible for the lack of congruence (Dincă et al. 2015). Note, however, that these problems do not appear to affect the remaining Lepidoptera data sets for which we observe high levels of congruence (five data sets). The picture is similarly confusing for fish (Actinopterygii) where low congruence is observed for the Genbank data set while marine fishes are doing well (three data sets). Similarly, inconsistent patterns are observed for Hymenoptera and Arachnida.

We suspect that these inconsistent results are largely due to the lack of rigorous studies that establish congruence with confidence. Such studies should be based on data sets where both morphological and DNA sequence information are obtained for all specimens belonging to a dense taxon sample. Subsequently, all specimens that cause conflict need to be re-examined in order to determine the proportion of specimens that were initially misplaced based on morphology and the proportion that were initially misplaced based on DNA barcodes (=pre-sorting error). Only then can one determine the number of specimen and species for which there is genuine conflict between the data sources. Such studies are unfortunately lacking. However, this lack of data should not affect our overall conclusions with regard to the effect of barcode length and position on congruence levels because morphospecies sorting errors affect both full-length barcodes and the mini-barcodes that were excised from the former.

But what is gained if all specimens were to be sorted based on barcodes and morphology? Using both sources of data would allow for higher quality work based on the principles of integrative taxonomy (Dayrat 2005; Schlick-Steiner et al. 2010), but it would not accelerate species discovery unless the morphological work would require less time. This is fortunately the case when pre-sorting with barcodes is adopted. This is shown in Wang et al. (2018) and Srivathsan et al. (2019), who pre-sorted 4000 ant and 7000 phorid specimens, respectively, with barcodes. Checking for congruence between mOTUs and morpho-species was fast because the specimens had already been pre-grouped into putative species based on barcodes. In addition, barcode distances provided an approximation for which putative species were closely related and should be compared. Lastly, for abundant species, only a subset of the specimens had to be studied with morphology. This subset could be selected based on genetic, temporal, and geographic gradients (Wang et al. 2018; Srivathsan et al. 2019); that is, the highly-skilled specialists could target a small proportion of specimens for dissection and slide-mounting.

Barcode Length and Species Delimitation Methods

We here tested the widespread assumption that mOTUs based on full-length barcodes are more reliable than those based on mini-barcodes (Burns et al. 2007; Min and Hickey 2007). If this assumption was confirmed, then the use of mini-barcodes for pre-sorting would have to be discouraged despite higher amplification success rates and lower cost. However, we find that the performance of *cox1* mini-barcodes with a length >200 bp do not differ substantially from the performance of full-length barcodes. Indeed, the full-length barcode only yielded the highest congruence values in 49 out of 182 tests (Fig. and Supplementary Fig. S6 available on Dryad); that is, some minibarcodes outperformed or matched the performance of the full-length barcodes in the remaining 133 tests.

We also find that the choice of species delimitation algorithm matters little as long as the mini-barcodes are longer than 200 bp (Fig. 4). This is fortunate as objective clustering and ABGD are less computationally demanding than PTP, which necessitates the reconstruction of ML trees. However, there are some exceptions. Firstly, when mini-barcodes are extremely short (~100 bp), ABGD and PTP tend to underperform relative to objective clustering. PTP's poor performance for 100-bp mini-barcodes is not surprising given that it relies on tree topologies which cannot be estimated with confidence based on so little data. ABGD's poor performance is mostly observed for certain priors (e.g., $P=0.04$: Supplementary Figs. S5 and S6 available on Dryad). Under these priors, ABGD tends to lump most of the 100-bp barcodes into one or a few large clusters. Prior-choice also affects ABGD's performance for full-length barcodes. Overall, ABGD does not perform well with very high priors ($P=0.04$: Supplementary Fig. S6 available on Dryad vs. $P=0.001$: Figs. 2 and) and we conclude that the selection of the best priors and/or clustering thresholds remains a significant challenge for the study of largely unknown faunas that lack morphological or other gene information that can be used as a criterion for selecting priors/thresholds. The literature suggests taxon-specific parameters, but we would recommend the use of multiple methods and thresholds in order to distinguish robust from labile mOTUs that are heavily dependent on threshold- or prior choices. The latter mOTUs should be targeted in reconciliation studies utilizing another type of data. As illustrated earlier, these reconciliation studies can focus on only 10–15% of all specimens that cause species-level conflict.

Barcode Length and Species Identification

Similar to our tests of species delimitation, we find that mini-barcodes >200 bp have no significant performance difference when compared with full-length barcodes. Full-length barcodes have only marginally—albeit statistically non-significantly—higher identification success rates than mini-barcodes >200 bp (Table 2: 0.00–4.15% difference, 0.37% median). This is consistent with the findings of Meusnier et al. (2008) who reported at least 95% identification success for 250-bp barcodes, while full-length barcodes had a success rate of 97%. Overall, our data suggest that mini-barcodes >200 bp are suitable for species identification. The poorer performance of shorter mini-barcodes (<200 bp) is largely due to an increase of ambiguous matches rather than incorrect matches. These mini-barcodes are less informative, which is probably due to their short length and the placement of many short barcodes in a more conserved part of the Folmer region (see below).

Positional Effects

Overall, mini-barcodes at the 3′ end of the Folmer region outperform mini-barcodes at the 5′ end. This is consistent across all three species delimitation methods and was also noticed by Shokralla et al. (2015a) for fish species. This positional effect is apparent when match ratios are compared across a "sliding window" (Fig. 2). The lowest congruence with morphology is observed for 100-bp mini-barcodes with midpoints at the 50, 110, and 170-bp marks. However, this positional effect is only significant when the barcode lengths are very short (<200 bp). Once the mini-barcodes are sufficiently long (>200 bp), there is no appreciable difference in performance, which is not surprising because sampling more nucleotides helps with buffering against positional changes in nucleotide variability.

These changes in nucleotide variability may have functional reasons related to the conformation of the Cox1 protein in the mitochondrion membrane. The Folmer region of Cox1 contains six transmembrane α-helices that are connected by five loops (Tsukihara et al. 1996; Pentinsaari et al. 2016). Pentinsaari et al. (2016) compared 292 Cox1 sequences across 26 animal phyla and found high amino acid variability in helix I and the loop connecting helix I and helix II (corresponding to position 1–102 of *cox1*), as well as end of helix IV and loop connecting helix IV and V (corresponding to positions approximately 448–498). These regions of high variability are distant from the active sites and thus less likely to affect Cox1 function (Pentinsaari et al. 2016). This may lead to lower selection pressure and thus higher variability in these areas which could impact the performance of mini-barcodes for species delimitation.

Accelerating Biodiversity Discovery and Description

In our study, we test whether species-level sorting with mini-barcodes is as accurate as discovery with full-length barcodes. However, the taxonomic impediment is also caused by the lack of taxonomic capacity for identifying specimens to species and describing new species. Fortunately, species-level sorting with barcodes can help with species identification because some specimens placed in mOTUs can be identified via barcode databases. Common species are more likely to benefit because they are more likely to have been barcoded. This is illustrated by our recent work on dragon- and damselflies (Odonata), ants (Formicidae), and non-biting midges (Chironomidae) of Singapore (Baloğlu et al. 2018; Wang et al. 2018; Yeo et al. 2018). These studies document why it is important to distinguish between species- and specimen-level identification rates because the results differ widely. For odonates, BLAST-searches identified more than half of the 95 mOTUs and >75% of the specimens to species. The corresponding numbers for ants and midges were ca. 2% and 10% at mOTU-level, and 9% and 40% at the specimen-level; that is, despite low mOTU identification success for midges, a fairly large number of specimens could be identified because very common species were already represented in a barcode database.

In addition, mOTUs based on sequences are also useful for studying biodiversity patterns because they can be readily compared across studies and geographical regions even if the species are not yet described (Ratnasingham and Hebert 2013). In contrast, new species identified based on morphology usually remain unavailable to the scientific community until the descriptions are published. This is a very significant difference because a large amount of downstream biodiversity analysis can be carried out based on newly discovered species before the latter are described. However, cross-study comparisons are only straightforward for mOTUs because sequences can be readily shared and compared while this is much more difficult with morphological illustrations. Pre-sorting specimens into mOTUs thus allows for the study of species richness and abundances before taxa are described.

Conclusions

We here illustrate that mini-barcodes are as reliable for pre-sorting specimens into putative species as full-length barcodes. Mini-barcodes are also suitable for identifying unidentified barcodes to species based on barcode reference databases. We would thus argue that mini-barcodes should be preferred over full-length barcodes because they can be obtained more readily for specimens that only contain degraded DNA (Hajibabaei et al. 2006) and are much more cost-effective. In particular, we recommend the use of mini-barcodes >200 bp at the 3′ end of the Folmer region. It is encouraging that such mini-barcodes perform well across a large range of metazoan taxa. These conclusions are based on three species delimitation algorithms (objective clustering, ABGD and PTP) which, overall, have no appreciable effect on the performance of barcodes. If the DNA of the specimens is so degraded that very short mini-barcodes have to be obtained, we advise against the use of PTP and ABGD (especially with high priors) in order to reduce the likelihood that morphospecies are lumped.

Supplementary Material

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.08kprr4zk.

Funding

This work was supported by a Ministry of Education grant on biodiversity discovery (R-154-000-A22-112).

Acknowledgments

We would like to acknowledge Athira Adom and Jonathan Ho for data processing, Lee Wan Ting for performing the molecular work, and Emily Hartop and Wendy Wang for commenting on the manuscript.

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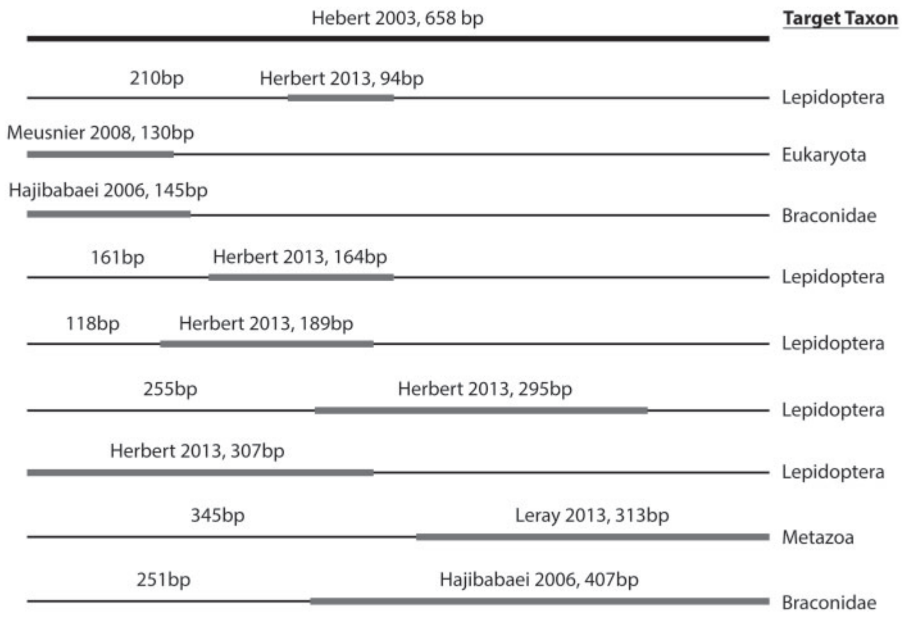
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Lepidoptera
255bp
Herbert 2013, 295bp
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Lepidoptera
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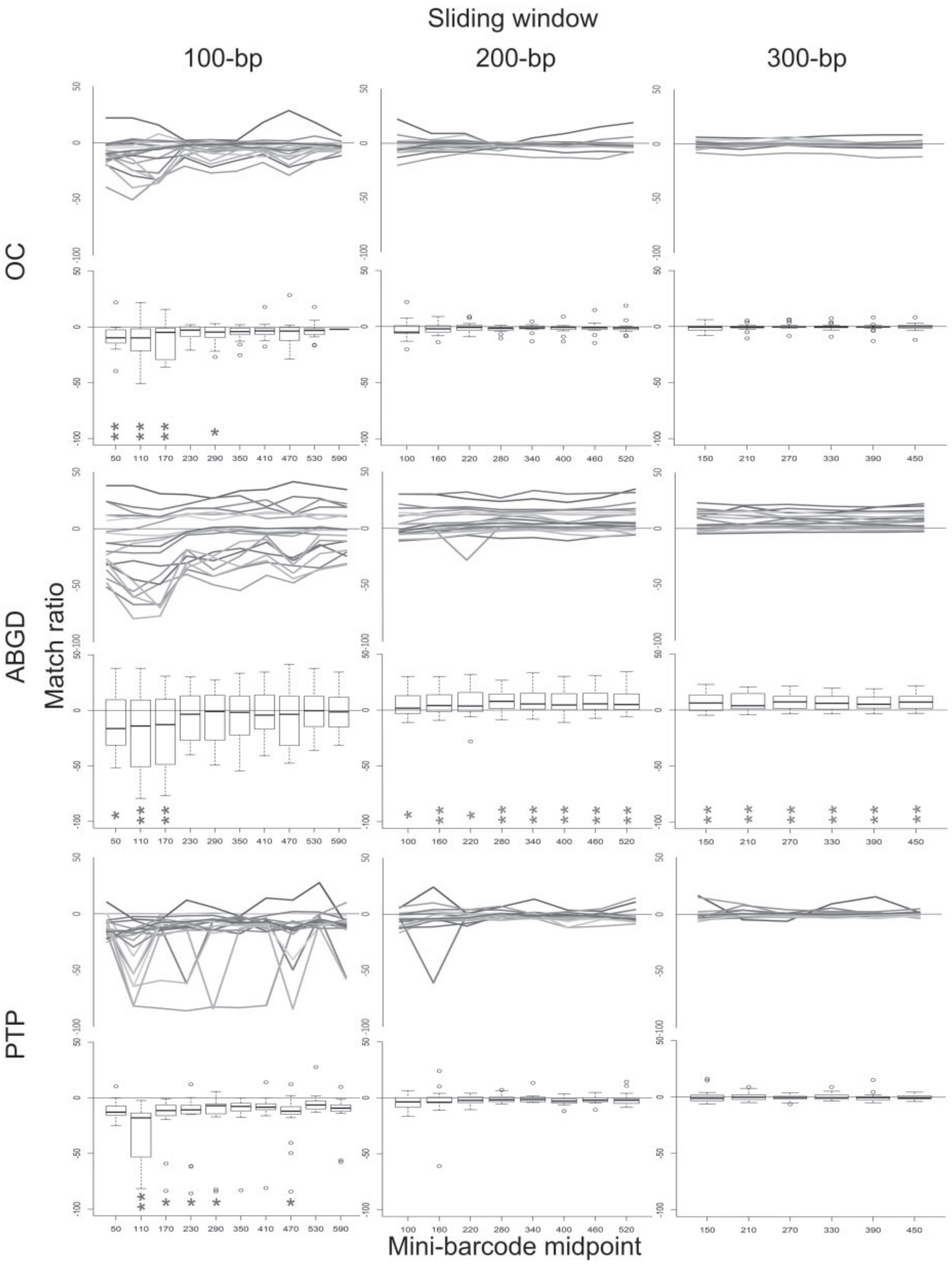
Hajibabaei 2006, 407bp
Braconidae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200-bp
300-bp
OC
ABGD
PTP
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0
-50
-100
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290
350
410
470
530
590
100
160
220
280
340
400
460
520
150
210
270
330
390
450
Mini-barcode midpoint

Objective clustering 2%

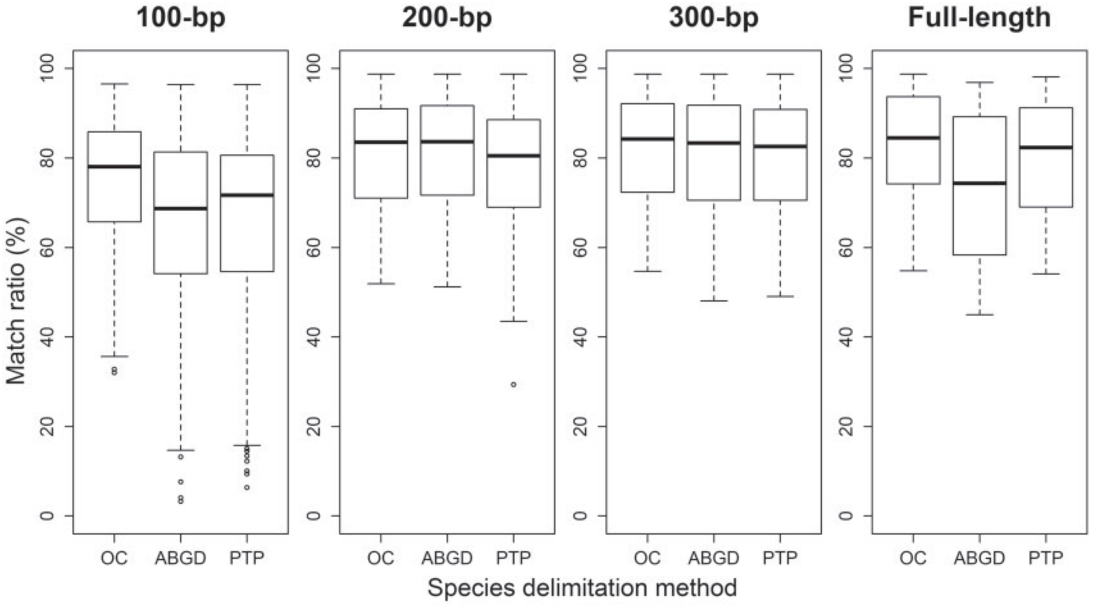
Length	94	130	145	164	189	295	307	313	407	657	Average
Midpoint	**257**	**65**	**73**	**243**	**213**	**405**	**154**	**502**	**455**	**329**	
20 Empirical Datasets											
Ecuadorian Geometridae	94	95	96	98	98	99	99	99	99	99	98
Great Barrier Reef Fish	94	91	92	95	95	97	94	97	97	97	95
Pakistani Lepidoptera	88	87	90	91	91	95	93	95	95	97	92
South China Sea Fish	92	86	88	94	94	94	94	94	94	95	92
South American Butterflies	83	89	88	90	90	92	93	92	93	93	90
Amazonian Moths	87	88	89	90	91	91	90	91	91	91	90
European Marine Fish	81	74	84	92	90	97	93	94	97	97	90
Canadian Echinoderms	81	89	85	91	88	91	91	91	94	95	90
North Sea Molluscs	77	85	84	90	90	89	92	84	85	87	86
German EPT	78	85	84	85	84	83	85	83	83	84	83
North European Tachinidae	81	81	82	81	83	83	83	83	83	84	82
North American Birds	84	77	77	82	81	85	82	84	85	85	82
German Araneae & Opiliones	69	77	76	79	78	80	82	82	80	81	78
Northwest Pacific Molluscs	72	74	73	77	77	78	77	77	78	78	76
North American Pyraustinae	61	56	64	69	77	74	75	78	77	84	71
Congolese Fish	63	61	60	68	70	72	66	70	71	70	67
French Guianan Earthworms	52	74	74	54	63	65	65	65	65	60	64
Iberian Butterflies	51	55	56	59	63	61	62	63	61	65	60
Ecuadorian Chrysomelidae	54	54	55	55	56	55	55	55	55	55	55
Tanytarsus	47	51	49	57	57	58	59	55	56	57	55
Consolidated:	**77**	**78**	**79**	**82**	**82**	**83**	**83**	**83**	**83**	**84**	**81**
6 Clade-based Datasets											
Coleoptera	76	78	78	80	80	80	81	80	80	81	79
Lepidoptera	72	73	74	77	78	80	79	80	81	81	78
Diptera	72	74	74	76	76	77	78	76	77	77	76
Arachnida	63	66	65	65	66	68	68	67	67	67	66
Actinopterygii	64	60	61	65	65	66	64	66	66	66	64
Hymenoptera	60	63	63	63	63	65	65	65	64	64	64
Consolidated:	**69**	**70**	**70**	**72**	**73**	**74**	**73**	**73**	**73**	**74**	**72**
Overall Consolidated:	**70**	**72**	**72**	**74**	**75**	**76**	**75**	**75**	**75**	**76**	**74**

ABGD P=0.001

Length	94	130	145	164	189	295	307	313	407	657	Average
Midpoint	**257**	**65**	**73**	**243**	**213**	**405**	**154**	**502**	**455**	**329**	
20 Empirical Datasets											
North Sea Molluscs	94	90	92	94	94	89	90	89	86	80	90
South China Sea Fish	92	78	83	89	93	94	92	93	91	90	89
Great Barrier Reef Fish	79	75	79	90	89	97	92	95	95	94	88
Canadian Echinoderms	87	78	76	91	91	87	91	95	92	89	88
Pakistani Lepidoptera	61	67	75	84	89	95	91	95	96	94	85
German EPT	85	84	85	86	87	83	85	84	78	67	82
Ecuadorian Geometridae	48	58	57	96	70	99	99	99	99	97	82
South American Butterflies	60	70	77	84	84	89	91	91	91	81	82
European Marine Fish	80	72	72	85	86	83	84	80	78	69	79
North American Birds	61	67	73	74	76	81	83	83	84	87	77
Amazonian Moths	72	43	42	88	75	87	90	90	91	89	77
North European Tachinidae	71	60	66	77	80	77	84	82	82	84	76
French Guianan Earthworms	78	89	93	81	81	73	80	70	59	55	76
Northwest Pacific Molluscs	76	75	75	77	77	76	77	76	76	63	75
German Araneae & Opiliones	76	18	79	85	82	71	74	73	65	52	67
Congolese Fish	68	44	55	67	71	70	63	67	71	65	64
Tanytarsus	67	71	71	61	62	50	56	48	47	45	58
North American Pyraustinae	25	29	41	67	76	70	74	67	58	59	57
Ecuadorian Chrysomelidae	55	55	54	56	56	57	57	55	57	58	56
Iberian Butterflies	26	36	42	53	57	60	65	59	55	52	50
Consolidated:	**69**	**63**	**71**	**80**	**79**	**80**	**82**	**81**	**79**	**74**	**76**
6 Clade-based Datasets											
Coleoptera	77	80	80	81	82	77	79	76	73	67	77
Diptera	69	73	74	76	77	73	75	72	69	63	72
Lepidoptera	42	54	62	74	77	79	78	79	77	71	69
Arachnida	71	71	71	69	68	66	64	64	64	57	67
Actinopterygii	59	54	55	64	64	66	66	65	65	64	62
Hymenoptera	62	64	65	64	65	62	63	62	59	55	62
Consolidated:	**63**	**66**	**68**	**72**	**73**	**71**	**72**	**71**	**69**	**64**	**69**
Overall Consolidated:	**65**	**65**	**68**	**74**	**74**	**73**	**74**	**73**	**71**	**66**	**70**

PTP

Length	94	130	145	164	189	295	307	313	407	657	Average
Midpoint	**257**	**65**	**73**	**243**	**213**	**405**	**154**	**502**	**455**	**329**	
20 Empirical Datasets											
Great Barrier Reef Fish	89	85	87	91	91	95	90	95	96	96	91
Pakistani Lepidoptera	95	83	82	87	90	95	95	95	95	94	91
Ecuadorian Geometridae	14	89	91	94	97	98	97	96	99	98	87
North Sea Molluscs	78	71	82	94	96	86	92	86	93	93	87
South China Sea Fish	86	75	77	87	88	92	89	89	92	92	87
Canadian Echinoderms	75	71	78	83	90	89	90	87	91	89	84
German EPT	82	80	81	81	83	79	83	82	79	80	81
North European Tachinidae	77	70	73	82	80	84	83	83	82	85	80
Amazonian Moths	32	88	38	89	88	91	89	91	90	90	79
German Araneae & Opiliones	72	73	77	77	79	82	80	77	81	79	78
North American Birds	72	67	66	77	78	81	82	81	85	85	77
French Guianan Earthworms	71	83	69	85	80	81	62	78	75	69	75
European Marine Fish	71	60	61	71	78	80	74	79	79	80	73
Northwest Pacific Molluscs	72	67	68	75	74	72	74	76	77	78	73
Congolese Fish	57	44	50	67	69	68	64	68	71	69	63
Tanytarsus	51	58	64	61	58	68	63	64	66	63	62
North American Pyraustinae	45	58	54	56	49	58	71	80	76	58	61
Ecuadorian Chrysomelidae	55	57	55	56	55	55	54	55	55	55	55
Iberian Butterflies	32	47	48	47	48	59	58	61	58	54	51
South American Butterflies	0	0	0	0	0	89	90	93	91	91	45
Consolidated:	**63**	**68**	**66**	**74**	**75**	**81**	**81**	**82**	**82**	**82**	**76**
6 Clade-based Datasets											
Coleoptera	74	73	73	78	78	81	81	80	81	80	78
Arachnida	69	70	70	70	69	70	68	68	68	66	69
Diptera	37	71	69	55	71	76	76	75	75	75	68
Lepidoptera	39	65	41	64	33	79	78	77	79	78	63
Hymenoptera	44	63	63	62	64	66	66	65	65	65	62
Actinopterygii	22	49	51	61	61	64	61	64	65	65	56
Consolidated:	**45**	**64**	**59**	**65**	**61**	**73**	**72**	**72**	**73**	**72**	**66**
Overall Consolidated:	**50**	**65**	**61**	**67**	**64**	**75**	**74**	**74**	**75**	**74**	**68**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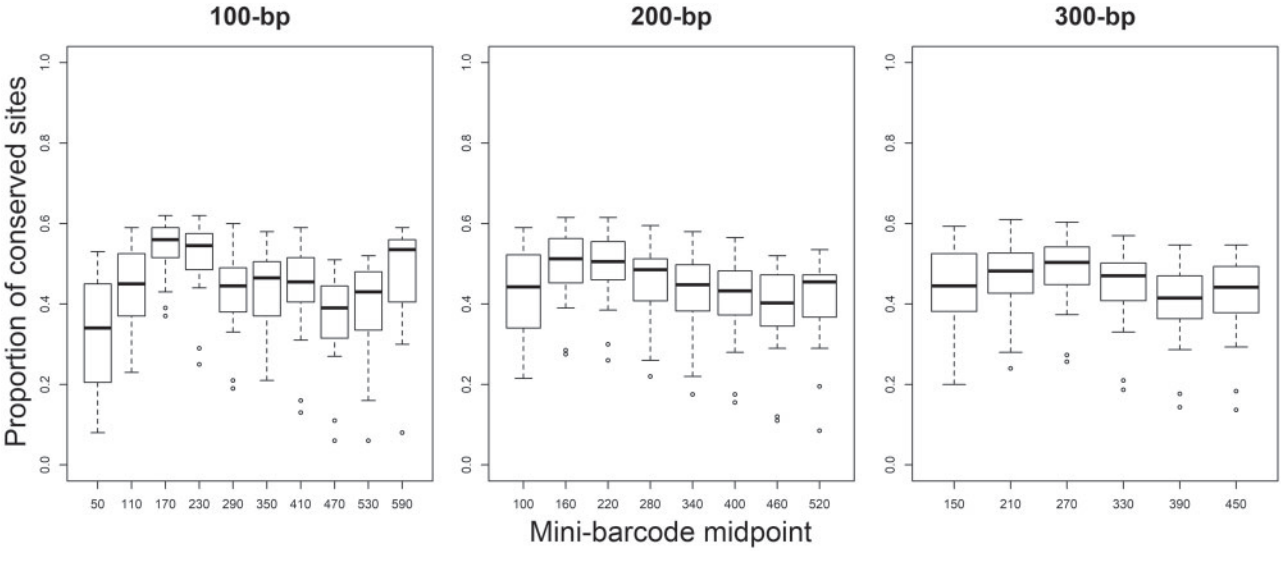
60
80
100
OC
ABGD
PTP
Species delimitation method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0.6
0.8
1.0
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290
350
410
470
530
590
100
160
220
280
340
400
460
520
150
210
270
330
390
450
Mini-barcode midpoint