What's in a name? Bioinformatics and the challenge of using eDNA metabarcoding to report non-indigenous species detections

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Abstract

Environmental DNA (eDNA) metabarcoding is increasingly employed for surveillance of non-indigenous species (NIS), offering the promise of simultaneous multi-taxon detection, scalability, and the capacity for early detection before populations become established. The sensitivity of metabarcoding that makes it attractive for NIS surveillance also creates a meaningful risk of erroneous detections and this risk is compounded by the extraordinary nature of NIS surveillance itself: The target signal, by definition, is not expected to be present. Here, we describe three validation criteria that practitioners should consider when evaluating their confidence in putative NIS detections from metabarcoding datasets. These are: (1) the strength of the detection signal; (2) the ecological plausibility of the detection; and (3) the confidence in the taxonomic assignment. We argue that the metabarcoding community working on NIS detection would benefit from a more deliberate and transparent approach to reporting—one that explicitly acknowledges the uncertainty surrounding any given detection and contextualizes it within this structured evaluation framework. We do not provide quantitative thresholds for confirming NIS detections or specify any explicit implementation of the proposed framework, as such determinations will depend on the ecological and management context and are appropriately made in dialogue between the scientists reporting detections and the managers who must act on them. That said, we believe that the considerations outlined here should be seriously considered by both data providers and end-users who intend to utilize eDNA metabarcoding to report NIS, particularly when such reporting might trigger management action.

Keywords: Environmental DNA, metabarcoding, biological invasions, non-indigenous species, surveillance, detection

1. Introduction

In a recent analysis of ballast water samples collected in the northeast Pacific Ocean using a standard COI metabarcoding locus, one of us noted 6 reads assigned to

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Homo heidelbergensis. From the perspective of an invasion biologist, this represented a rather notable observation, as Europe is generally recognized as the species' native range (Trájer, 2024). Also, notwithstanding certain fringe hypotheses (e.g., Verhaegen, 1985), *H. heidelbergensis* is widely considered to be terrestrial in habit (Stringer, 2012). Possibly most remarkable is the fact that the species is thought to have gone extinct roughly 200,000 years ago (Raia et al., 2020). Given that extraordinary claims require extraordinary evidence, and assuming that the discovery of extant marine populations of ancient hominids would count as extraordinary (although possibly not entirely unprecedented; see Rossi, 2015), we pursued more detailed analysis of this putative species-level assignment. Perhaps fortunately, although *H. heidelbergensis* was returned by our bioinformatic algorithm as the most likely assignment, confidence was low and manual blastn searches retrieved reference sequences with similar identity and coverage belonging to a congener—*Homo sapiens*—that we consider much more likely to have contributed DNA to a ballast water sample collected in 2017.

It is possible that we are not the only group to have detected putative *H. heidelbergensis* sequences in contemporary environmental samples; such things are rarely reported in the literature. It is certainly the case, however, that we are not the only group to have detected, with some frequency, sequences of species that we do not expect to be there. This is a challenge for any metabarcoding study. But it is a particular challenge for metabarcoding studies engaged explicitly in the business of detecting organisms that one does not expect to find in one's samples—namely, introduced non-indigenous species (NIS).

eDNA metabarcoding is increasingly being used for monitoring and detection of NIS, particularly across aquatic habitats (Borrell et al., 2017; Brown et al., 2015; Darling et al., 2018; Pappalardo et al., 2025). Amplification of an informative locus from a swath of taxa within a community can provide a cost-effective approach to monitor for a wide range of invasive species from a relatively limited number of samples. Critically, the approach can also be piggy-backed on non-targeted monitoring efforts aimed at more general biodiversity endpoints (e.g. indicators of community health or aquatic ecosystem integrity), essentially picking up NIS detections as "by-catch" in these broader surveys (Fonseca et al., 2023). These community-based genetic methods are considered advantageous over traditional morphological approaches for many reasons such as the ability to identify early life history stages and cryptic species (Lira et al., 2023), lower resource requirements (Iacaruso et al., 2026), decreased risk in challenging sampling environments (Iacaruso et al., 2026), higher sensitivity at low population densities (Wilcox et al., 2016), and reduced need for deep taxonomic expertise for each group of organisms encountered (Pappalardo et al., 2021). Early detection of NIS has been touted as one of the central potential benefits of these methods, and metabarcoding has been applied to NIS surveillance across a wide range of systems—from ports and harbors (Brown et al., 2016; Lavrador et al., 2024; Pearman et al., 2021) to ballast water (Li et al., 2025; Pagenkopp Lohan et al., 2022), zooplankton communities (Pappalardo et al., 2025; Van Nynatten et al., 2023), and insect biosurveillance (Batovska et al., 2021; Piper et al., 2019). Indeed, there is now a growing body of evidence that metabarcoding regularly detects NIS that conventional surveys miss, and in some cases has achieved detections that preceded physical capture of target organisms (Couton et al., 2022; Holman et al., 2019; Jeunen et al., 2022; Li et al., 2025).

These applications are not without risk. Like all monitoring approaches, metabarcoding is susceptible to error. False positives and false negatives can arise through a multitude of different pathways, from sampling in the wrong locations or at the wrong times to amplifying too short a DNA fragment or using a reference database that contains mis-assigned sequences. At the biological level, environmental samples contain an inherently noisy mixture of DNA from organisms present at the time of collection, including organisms that recently died at or near the sampling location (e.g., Merkes et al., 2014), and organisms whose genetic material was transported from elsewhere in the catchment or water body (e.g., Inoue et al., 2023). At the laboratory level, contamination during sample collection, preservation, extraction, and amplification can introduce exogenous DNA, and the polymerase chain reaction itself can generate chimeric artifacts—sequences that are hybrids of two or more genuine template sequences—that can superficially resemble novel taxa (Ficetola et al., 2016; Keck et al., 2023). At the bioinformatic level, taxonomic assignment algorithms must identify each sequence as the best match to some entry in a reference database, and the quality of that assignment is only as good as the quality and completeness of the database itself. The issue of reference database completeness and accuracy is an abiding concern in the field, so much so that Keck et al. (2023) identified seven distinct challenges—including mislabelled sequences, sequencing errors, taxonomic conflicts, and missing taxa—any of which can produce error. Reference databases for many taxonomic groups, and particularly for the marine invertebrate and zooplankton assemblages that are among the most important contributors of aquatic NIS, remain fragmentary (Bachmann et al., 2023; Weigand et al., 2019), and errors or misidentifications in existing entries propagate directly into erroneous detections, sometimes in ways that are difficult for end-users to identify.

All these considerations must be addressed in potentially fraught decision-making contexts. In contrast to many general biodiversity assessments, the use of metabarcoding for invasive species detection can have specific, tangible, and often fast-moving consequences when a potential invader is detected in a novel location (Darling et al., 2020). The actions that managers are required to take to quickly respond to a novel invader are expensive and time-consuming, leaving them with a genuine concern about the degree of certainty that metabarcoding from an environmental sample provides. Both false positives and false negatives can have devastating economic consequences, though in different ways. Furthermore, a pattern of reporting errors can erode the credibility of monitoring programs with the management agencies that fund them (Mahon et al., 2023). Thus, from a management perspective, methodological approaches that allow managers to capture and look at the NIS in question may sometimes be considered "safer" even if they bias monitoring programs toward higher false negative rates (Darling et al., 2020).

It is with these concerns in mind that we offer this perspective, as we believe the best approach to building trust in metabarcoding methods is to transparently recognize the uncertainties associated with detections, particularly in reporting contexts with potentially costly triggers for management action. In this paper, our aim is to identify ways in which we can improve such reporting—specifically, to highlight what components of data can and should be used to determine overall confidence in a NIS detection. We acknowledge that many aspects of study design and laboratory workflow for detecting invasive species have been covered extensively in previous guidance documents (Klymus et al., 2024; Shaffer et al., 2025; Watts et al., 2025), and we do not revisit those here. Rather, we describe a set of considerations organized around three validation criteria that practitioners might bring to bear when evaluating whether a putative NIS detection in a metabarcoding dataset is likely to be genuine. Our goal is not to provide an algorithm for automated decision-making, but rather to make explicit the kinds of reasoning that experienced practitioners already apply, if often implicitly, in the hope that naming these considerations will make them easier to communicate, document, and ultimately implement in decision-making contexts.

We wish to be explicit about what this paper does not do. We are not outlining the specific steps needed to: (1) defend the quality of sequence data generated, which has been covered by existing minimum information standards (e.g., Klymus et al., 2024) and can also affect species identification; (2) identify quantitative thresholds for when to say NIS DNA has been detected; or (3) make claims about risk management based on a positive detection. We do not claim that any level of confidence in any eDNA-based detection is sufficient to establish the physical presence of a NIS in a sampled environment, and we do not generally specify minimum read counts, sequence similarity scores, or other numerical criteria for classifying a detection as confirmed. Future attempts to implement the framework outlined here in operational settings will need to confront these issues, and much will depend on the ecological and management context, appropriately determined in dialogue between the scientists reporting detections and the managers who must act on them (Mahon et al., 2023).

2. The Three Validation Criteria

Conceptually, two distinct but equally important questions must be answered for any taxonomic name appearing on a metabarcoding inventory: First, is there really something there? Second, is it really what we're saying it is? The first question is somewhat easier to address than the second, though both have an element of art to them that make establishing thresholds for decisions or creating algorithmic approaches to solutions challenging.

Here, we propose three validation criteria for assessing positive detections that ought to be evaluated to support reporting NIS (or, for that matter, any other species of concern) from metabarcoding datasets (Table 1). To address the question of whether there is really something there worth reporting, validation criterion 1 addresses the question "How strong is the signal?" Validation criteria 2 and 3 address the thornier issue of whether the thing being detected is what we claim it is, asking both "Does the detection make sense?" and "How confident are we in the taxonomic assignment?" Careful attempts to answer these questions can help critically evaluate detections of species of concern, where management decisions cannot be taken lightly. Below, we discuss the three validation criteria and provide some practical suggestions for approaching them.

Table 1. The Three validation criteria for determining confidence in NIS detection.

Validation Criterion	Primary factor	Secondary factor	Notes
How strong is the signal?	Number and abundance of reads	Raw read abundance	Rare reads can be biologically real but are stochastic; common reads are strongly credible. Counts alone are not proportional to abundance.
		Sequencing depth	Deep sequencing lowers detection limits for rare taxa; signal must be interpreted relative to depth.
		Relative read abundance	May be more informative than raw counts; thresholds should scale with read depth.
	Replication	Technical replication	Replicable detection greatly increases confidence
		Spatial replication	Multi-site detections are more credible than single-site occurrences
		Temporal replication	Recurrence across seasons or years strongly supports true presence
	Genetic diversity	Intraspecific variation (multiple ASVs)	High diversity is likely to represent a genuine population of the target and is unlikely in cases of spurious detections.
Does the detection make sense?	Biological plausibility	Environmental matching	Large mismatches (depth, salinity, habitat type) raise concern, but tolerances can shift post-invasion.
		Geographic matching	Mismatches heighten scrutiny, but databases are incomplete and recent or cryptic invasions may lack records.
	Introduction likelihood	Association with pathways and vectors	Shipping, aquaculture, and hydrologic connectivity increase plausibility; isolated systems require stronger evidence.
		Spatial patterning	Detection concentrated near ports or corridors supports biological realism.
		Prior regional records	Morphological records may carry more weight than prior eDNA-only reports.
	Other	Alternative signal sources	Airborne DNA, downstream transport, symbiont-driven misassignment, etc.
How confident are we in the taxonomic assignment?	Taxonomic representation	Contextual relevance of reference data	Regional sequences improve local accuracy, but global databases are required to enable novel invader detection.
		Reference database quality	Incomplete or mis-curated references are a dominant source of error
		Reference database coverage	Multiple sequences per species and good coverage of close relatives needed.
	Phylogenetic consistency	Phylogenetic placement	Placement in a supported monophyletic clade is stronger evidence than pairwise similarity alone.
		Barcode gap presence	Clear inter- vs. intraspecific divergence supports species calls; absence of a gap warrants caution.
	Bioinformatic choices	Pipeline robustness	Detections using different pipelines may boost confidence.
		Reference database	Positive detections across multiple reference databases increase support.
	Marker choice	Marker-specific limitations	Taxonomic confidence must be evaluated considering marker evolution rates and reference completeness.
		Multi-marker replication	Cross-marker detection is a powerful confirmation

2.1 *Validation Criterion 1: How strong is the signal?*

A natural intuition when evaluating a putative detection in metabarcoding data is to look at the raw or relative number of reads. We do not think that this intuition is entirely unwarranted, although it should be followed with some caution. Raw read abundance is in many ways an artifact of stochastic processes—DNA extraction, PCR amplification, and sequencing—rather than a direct reflection of biological abundance (Kelly, 2022; Shaffer et al., 2025). Because sequencing reads represent a vanishingly small fraction of the amplicons generated in a PCR reaction, and because rare amplicons are therefore sampled stochastically and inconsistently across technical replicates, raw read abundance for a rare taxon does not necessarily provide reliable information about whether the taxon is genuinely present (Kelly, 2022). This suggests that rare reads should not be reflexively discarded as meaningless; they may, in fact, carry true biological information of potentially great value. However, we believe that the intuition to trust in the genuine presence of organisms represented by very common reads is warranted. Basically, rare reads—even singletons—*may* be biologically meaningful, but common reads *almost certainly* are (see caveats below).

Sequencing depth—the total number of reads generated per sample—is itself a critical variable in interpreting signal strength, one that has grown substantially more consequential as high-throughput platforms have become more common for metabarcoding. Some instruments can generate millions of reads per sample in a single run, a level of output that materially alters the detection landscape relative to shallower sequencing approaches. At very high sequencing depths, the stochastic barrier that typically prevents rare amplicons from appearing consistently is partially lowered. Namely, a taxon present at genuinely low template abundance in a sample may yield hundreds or even thousands of reads in a deeply sequenced library where it might yield zero or one in a shallower run. This has direct implications for the sensitivity of NIS detection. Early-stage invasions, at the point of greatest management value to intercept, are likely to be present at extremely low densities and therefore at very low template concentrations in environmental samples. Deep sequencing raises the probability that such a signal clears a detection threshold, and practitioners working with platforms capable of high read depths should recognize that the sensitivity floor of their survey is a function of sequencing depth as much as of sampling design or DNA extraction efficiency.

This sensitivity advantage, however, comes with an important corollary for evaluating signal strength. Deep sequencing can amplify the detectability of genuine signals and spurious ones by the same mechanism. A signal that would fall below detection in a library of 100,000 reads may reliably accumulate several hundred reads in a library of 50 million, making it appear far more substantial in absolute terms than its biological significance warrants. The practical implication is that raw read counts must always be evaluated relative to the total sequencing depth of the library. Relative read abundance—the proportion of total reads assigned to the putative NIS—may also be an important metric to consider, because it contextualizes the signal against the scale of the sequencing effort. When comparing detections across studies or datasets that differ substantially in sequencing depth, the effective sensitivity threshold differs between them, and a detection that barely exceeds the noise floor in a deeply sequenced dataset may be categorically different from one that does so in a shallow library, even if the raw read counts appear similar. Sequencing depth should therefore be reported alongside read counts as a fundamental parameter for evaluating and comparing signal strength.

Note that in the preceding we use vague thresholds—”rare” vs. “common” reads, “low” vs. “high” sequencing depth, the “noise floor” for detection, etc. We intentionally have avoided the issue of setting these thresholds more explicitly, largely because we feel that doing so requires consideration of both the monitoring context and the risk tolerance of the end-user, and it is therefore exceedingly difficult or even impossible to provide general thresholds. As a matter of practice, almost all users filter out singletons (detections represented by only a single read across an entire dataset) and some use relative abundance thresholds. Additionally, many users require a minimum number of total reads per sample (e.g., 10,000) to consider a run acceptable for further analysis. However, end-users highly sensitive to the risk of false negatives may opt for much lower thresholds, particularly for “high risk” watchlist or other priority species; thresholds that may be useful for general analyses of biodiversity (e.g. eliminating singletons and entire samples falling below a certain threshold) may not be acceptable in decision-making contexts depending on the risk tolerance of the end-user.

Rather than focusing on the number of reads—especially given the complexities noted above—a more robust indicator of a genuine detection is replicated occurrence across independent PCR reactions from the same extract (i.e., technical replication) or across independent samples collected in the monitoring effort (i.e., biological replication). The probability that an identical contamination event, sequencing error, or stochastic artifact should manifest independently in multiple technical replicates is low, and a species detected consistently across replicates is substantially more likely to represent a true biological signal than one appearing in a single reaction (Ficetola et al., 2016; Shirazi et al., 2021). PCR replicates of the same field sample may be used to estimate the probability that a taxon’s presence is genuine, rather than treating any single detection as indicative of presence (Ficetola et al., 2016; Fukaya et al., 2022). In reporting, these considerations may take many forms. For instance, Lilli et al. (2025) generated data from 9 technical replicates and reported the fraction of positive detections for each species of concern observed in the resulting inventories. Alternatively, thresholds for reporting might be chosen in coordination with decision-makers, recognizing the degree to which those end-users might tolerate false negative vs. false positive detections.

Spatial replication across independent field samples within a survey provides an analogous and possibly even more compelling form of confirmation. A NIS detected at multiple sites is far more credible than one detected at a single location (Pappalardo et al., 2025). Indeed, evidence based on biological replicates is the basis for emerging methods for occupancy modeling of metabarcoding data (Fukaya et al., 2022; Macé et al., 2025; McClenaghan et al., 2020). Such methods may also account for the considerations mentioned above, incorporating both read abundance and sequencing depth to infer overall confidence in detections (Fukaya et al., 2022; Macé et al., 2025). Not only do such patterns speak to the confidence in detection of the NIS in question, they also may help end-users to understand spatial patterns of population distribution, which may be crucially important information for decision-making. Ideally, formal occupancy modeling based on rigorous biological and technical replication would provide a strong foundation for assessing strength of signal in metabarcoding datasets. Practically, many users may adopt a less formal approach.

Temporal replication provides yet another dimension of signal strength. Longer-term temporal persistence strengthens inference: annual and seasonal surveys have demonstrated that genuinely present taxa recur across sampling periods, whereas spurious detections tend not to (Fukaya et al., 2022; Pukk et al., 2021). In a multi-year survey of 22 lakes in Michigan, Pukk et al. (2021) detected nine aquatic invasive species via eDNA metabarcoding over three consecutive sampling years (2016–2018). Species detected consistently across multiple lakes and years—such as *Cyprinus carpio* and *Neogobius melanostomus*—were interpreted with high confidence as established populations, whereas species detected sporadically at single sites or time points were flagged for further investigation (Pukk et al., 2021). Pappalardo et al. (2025) similarly found substantial temporal variation in zooplankton NIS detections in Alaskan ports, with some species of concern detected in multiple years and locations and most only once, highlighting the importance of repeated sampling to distinguish genuine but temporally variable presence from one-off detection.

The interpretation of temporal and spatial detection patterns should, however, consider known variation in eDNA persistence and transport across environments (Pont, 2024). In lotic systems, eDNA can be transported downstream over distances ranging from a few hundred meters to several tens of kilometers, depending on discharge, temperature, and particle dynamics (Pont, 2024). In marine and estuarine environments, tidal mixing, stratification, and complex current patterns can redistribute eDNA across spatial scales that do not correspond neatly to the footprint of a single sampling station (Andruszkiewicz et al., 2019). These dynamics mean that a detection in a high-flow or tidally dynamic system may reflect an organism located some distance away, while detection at the same location across multiple independent sampling events, when current conditions differ, provides stronger evidence of local presence. eDNA signals can fluctuate substantially over time, sometimes disappearing within hours despite confirmed presence of the source organism—underscoring why the persistence of a signal across repeated surveys carries substantially more evidential weight than detection in a single sample.

One other consideration when assessing strength of signal is the potential presence of multiple genetic variants of a single target species. While many species may be represented by a single amplicon sequence variant (ASV; also referred to as exact sequence variant, or ESV), possibly even at high read abundance, there are cases where a target comprises multiple ASVs, suggesting the presence of intraspecific genetic variation at the sampling site. Such variation provides additional support for validated detections, and should be included in assessments of confidence for reporting.

2.2. Validation Criterion 2: Does the detection "make sense"?

Fundamentally, NIS surveillance—and especially surveillance aimed at early or first detections of newly arrived species—involves searching for something that is, by definition, not supposed to be there. This inverts the typical logic of biological detection.

Whereas a wildlife ecologist might report the detection of a rare resident species with some confidence based on a single observation, the invasion biologist is immediately confronted with the question of whether the observation is genuine. This reflects differing baseline assumptions; the former is generally looking for something that belongs in the area but may not be there, whereas the latter is looking at something that doesn't belong there and may nevertheless be present. Despite the challenge, it would clearly be a mistake to reflexively dismiss all unexpected detections as artifactual. Non-indigenous species do arrive, and every impactful, established invasive species was once an unexpected newcomer.

Determining whether an eDNA metabarcoding detection is ecologically plausible begins with assessing whether the species' habitat requirements match the sampled environment. Implausible detections—such as marine taxa appearing in freshwater habitats, or terrestrial species showing up in deep-sea samples—might immediately be flagged as highly unlikely and raise concern for contamination, transport of exogenous DNA, or misassignment driven by incomplete reference databases (see below). There are various databases available to assist users in evaluating whether the habitat match is reasonable. For instance, for marine taxa, relevant ecological factors can be drawn from resources such as SeaLifeBase (Palomares & Pauly, 2026) for marine ranges and depth distributions, and WoRMS (Ahyong et al., 2026) for verifying habitat type. SeaLifeBase and WoRMS have programmatic access available through the *rfishbase* (Boettiger et al., 2012) and *worms* (Chamberlain & Vanhoorne, 2023) R packages respectively. Even more compelling evidence could be derived from explicit invasion risk assessment models based principally on environmental matching of potential recipient environments with conditions in species' native ranges (Chai et al., 2016; Coulter et al., 2022; Vilizzi, 2026; Vilizzi et al., 2021).

Ecological implausibility must be assessed cautiously, as numerous examples exist of NIS establishing outside their predicted environmental tolerances. For instance, zebra and quagga mussels of the genus *Dreissena* famously established in regions of North America recognized as likely outside of their tolerance based on mineral requirements for growth (Whittier et al., 2008). Evolutionary consideration also suggests the possibility of NIS appearing in regions that would appear unlikely yet represent habitats recently colonized after rapid adaptive change in introduced populations (Lee, 2023). The growing literature describing genomic and transcriptomic analysis of such shifts suggests that not only do NIS show up in places they don't belong *geographically*, they may show up in places they ought not to be *ecologically* (Tepolt, 2015; Viard et al., 2016).

Plausibility of detection must obviously be based not only on environmental factors, but also on geography. By definition, this is a problem exclusive to novel NIS, whose recognized global distributions will be non-overlapping with the sampled region, though for known established NIS the problem essentially dissolves, as the question of ecological plausibility has already been answered. Fortunately, availability and accessibility of distributional data is broad and rapidly growing. Databases such as the Global Biodiversity Information System (GBIF.org) and others provide massive general sources of distributional data on species across the tree of life. In addition, a number of NIS-specific databases exist that track global occurrences of species outside of their native ranges, such as the National Estuarine and Marine Exotic Species Information System (NEMESIS, https://invasions.si.edu/nemesis/; Fofonoff et al., 2022) for marine and freshwater taxa and Nonindigenous Aquatic Species (NAS, https://nas.er.usgs.gov/) databases for North America, the AquaNIS database in Europe (https://aquanisresearch.com/), and the World Register of Introduced Marine Species (WRiMS, https://www.marinespecies.org/introduced/). These data can be queried to assess the plausibility of a novel detection of a species in a new location; such plausibility obviously increases if said species is known to have been introduced elsewhere in the world, particularly if it has been introduced to the same continent, ocean basin, or region in which it is being detected.

For species that would represent truly novel observations outside their documented global distribution, the plausibility of detection also depends on whether there is any known mechanism by which that species may have arrived in the sampled region. There are well-known anthropogenic vectors of species introduction such as ballast water and hull fouling in maritime traffic, aquaculture transfers, exotic pet trade, hitchhiking in shipping containers, movement of infrastructure, and a host of “natural” mechanisms such as long-distance dispersal via rafting (on both natural and anthropogenic substrates) that are capable of transporting species well beyond their existing distributional limits (Ruiz et al., 2011). In many cases there may be known associations between such vectors and previous introduction events for particular species (e.g. ballast water transport and recreational boating for the aforementioned zebra and quagga mussels; Roberts, 1990; Schneider et al., 1998). In other cases, it may only be possible to recognize the credibility of such associations; for instance, coastal marine benthos, particularly broadcast spawners, are frequently entrained in ballast water (Briski et al., 2012), so their ability to move across ocean basins in ballast tanks must be taken seriously. Common pathways of introduction must also be considered, as global economies often drive establishment of trade routes, sometimes connecting disparate regions with sufficient climatic similarity to enable frequent and sustained exchange of biota (Ricciardi & MacIsaac, 2000). The availability of such transportation corridors and vectors of introduction bolster the plausibility assessment. Conversely, detections in isolated or poorly connected systems should invite greater scrutiny.

Perhaps the most powerful evidence for a detection is prior reports of the detected species in the sampled region. In many cases, eDNA studies have demonstrated their ability to extend known invasion ranges for non-indigenous species, detecting taxa beyond the limits of traditional surveys based on morphological characters (Couton et al., 2022; Jeunen et al., 2022). Prior records, even if sparse, increase biological plausibility by indicating an established or emerging invasion pathway. In contrast, detections lacking any historical precedent require more thorough investigation. While occurrence databases now include detections from eDNA data (e.g., gbif.org/metabarcoding), practitioners should consider giving greater weight to previous detections from traditional surveys using morphological data (e.g., Pappalardo et al. (2025) provided R code to filter occurrence records based on data type) to avoid second-hand verification from another molecular source, which may itself be troubled by similar uncertainties.

Examples from the literature illustrate how these considerations operate in practice. Jeunen et al. (2022) detected the Chinese sleeper (*Perccottus glenii*) via eDNA metabarcoding further west along the Dniepr River in Belarus than any previous ichthyological survey had recorded it. Rather than dismissing the detection as an artifact, the authors judged it ecologically plausible: *P. glenii* is a known invasive in the broader region, the Dniepr River system provides a continuous aquatic corridor for range expansion, and shipping traffic between river segments constitutes a recognized anthropogenic vector. Pearman et al. (2021) similarly found that NIS in Tahitian harbors were detected preferentially at sites with direct exposure to international shipping routes, consistent with biofouling-mediated introduction—a spatial pattern that would be difficult to attribute to laboratory or bioinformatic artifact.

Alternative explanations for the presence of a detected species' DNA should be pursued if the true presence of that species is found to be ecologically implausible based on the above criteria. Westerduin et al. (2023) detected DNA of several non-local Lepidoptera species in terrestrial eDNA samples from the Netherlands—species with no known populations in the region and no obvious anthropogenic introduction pathway. Rather than reporting these as genuine range expansions, the authors investigated potential mechanisms for the genetic signal, including airborne transport of pollen, grass, or shed scales, and horizontal gene transfer via the endosymbiont *Wolbachia*, which is known to promote mitochondrial introgression between Lepidoptera species and could cause a COI sequence to be assigned to the wrong taxon entirely. This example is a valuable reminder that the detection of a DNA sequence is not synonymous with the detection of an organism, and that ecological context should shape not only whether a detection is trusted but what questions are asked of it.

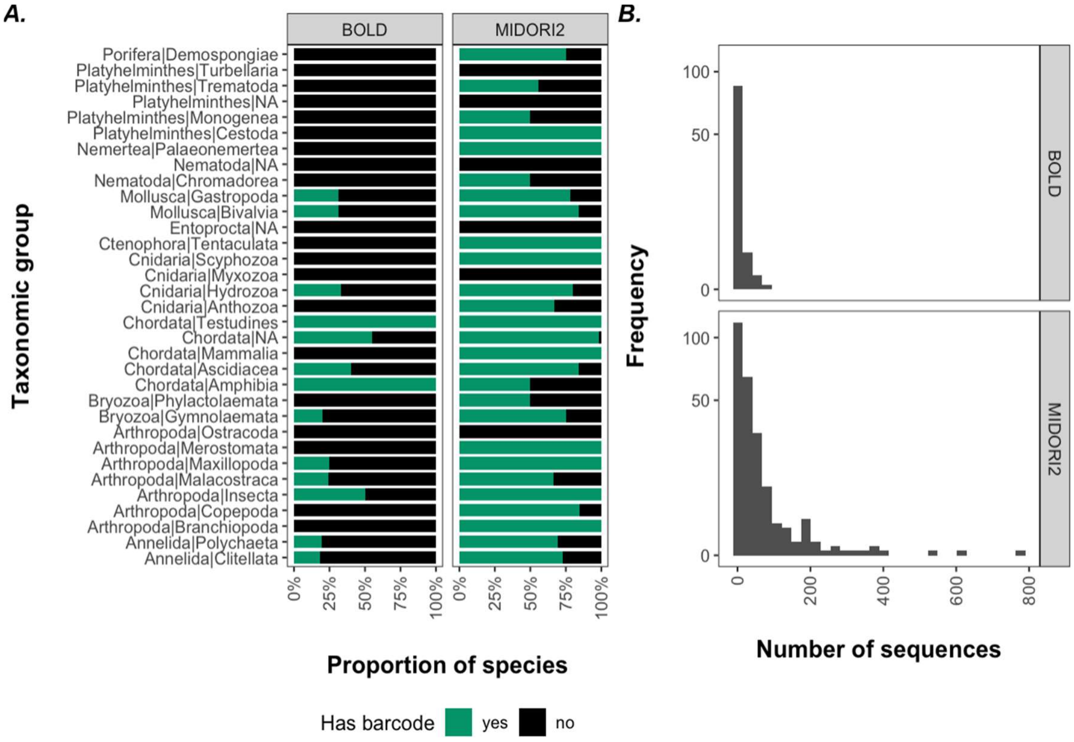
These considerations all suggest that there is a difference between detecting polar bear eDNA in a Colombian freshwater stream and detecting European green crab eDNA in an estuary in southeast Asia. Neither belong there, but the latter is plausible in all the ways that indicate it may require reporting—and quickly. Still, one may be left with the question: Why did I get polar bear in my metabarcoding inventory of a Colombian freshwater stream?

In theory, the reasoning that we apply here to potential detections of NIS could be applied to *all* taxa detected. In practice, without automated approaches to doing so, this likely represents an undue burden on reporting. But conceptually, there should be an explanation for why every name appears in one's metabarcoding inventory. Assuming the signal is real—that the sequence is derived from eDNA actually shed by an organism in the sampled environment—there are several possible explanations for the name associated with that sequence appearing on the inventory. Most obviously, it is because that species is a permanent or transient native resident of that system. In other words, it "belongs" there. Less obviously, it may be because it has been introduced to the system somehow and represents a non-native addition (again, even if transient) to the native fauna. Most troublingly, it could be because it is simply an error; the polar bear isn't really a polar bear, it is something else being identified as a polar bear with the bioinformatic algorithm. Our hope is that most of these cases could be resolved on deeper inquiry, and that exploration of the reference data would reveal such cases rather readily. This brings us to the final validation criterion.

2.3. Validation Criterion 3: How confident are we in the taxonomic assignment?

The third validation criterion—and in many ways the most technically demanding one to evaluate—is confidence in the taxonomic assignment of the detected sequence. For most detections, verifying that a particular taxon is non-native requires a species-level binomial to be assigned with high confidence to a sequence. Even a very strong signal may be an assignment of genuinely present DNA to the wrong taxon. Confidence in species-level calls rests principally on two foundations: the coverage and curation quality of the reference database for the focal taxon and its close relatives, and the resolution of the chosen marker to make species-level assignments. Incomplete or error-prone libraries are a primary source of spurious or ambiguous identifications. Even though species of concern may have better representation in reference databases for some taxonomic groups (e.g., fish; Marques et al., 2021), there are still many known introduced species without available barcodes, making detection using metabarcoding difficult or impossible (Fig. 1). In some instances, adding sequences from common, regional or local taxa can greatly increase the taxonomic assignments of the sequences from that area (Gold et al., 2021; Pappalardo et al., 2021), though using only a regional database for assessing taxonomic assignments may limit ability to detect novel invaders, as they are not expected to be present.

Figure 1. Representation of species introduced to the United States West Coast in reference databases. A. Proportion of introduced species that have a COI sequence available in BOLD (left panel) or MIDORI2 GB268 (right panel) for different taxonomic groups (Phylum | Class). B. Number of sequences available in each database for each of the Introduced species that have a barcode. Introduced species were identified from the SERC National Exotic Marine and Estuarine Species Information System (NEMESIS) database (Fofonoff et al. 2018). The United States West Coast included California, Oregon, and Alaska.



It is difficult to say what degree of representation in reference databases is sufficient for confident taxonomic assignment, but it is almost certainly not reflected in the common understanding of what it means for a species to be "barcoded." The deposition of a single sequence associated with a species–even a high-quality sequence attached to a vouchered specimen–is insufficient to support a confident assignment in most cases. Ideally, the reference data must include enough sequences to fairly represent the genetic variation present within each population of the target species, along with similar representation of variation present in closely related species, typically the target's congeners. Thus, successful "barcoding" of a species–the ability to distinguish that species from others in the database, given the reality of intraspecific genetic variation–likely requires dozens of sequences, minimally. To be frank, this rarely happens. For example, looking across 352 North American marine NIS (Fig. 1) in the Barcode of Life Database (BOLD) for COI, 26 of those species (7.4%) are represented by only a single sequence, and 67 (19%) by five or fewer.

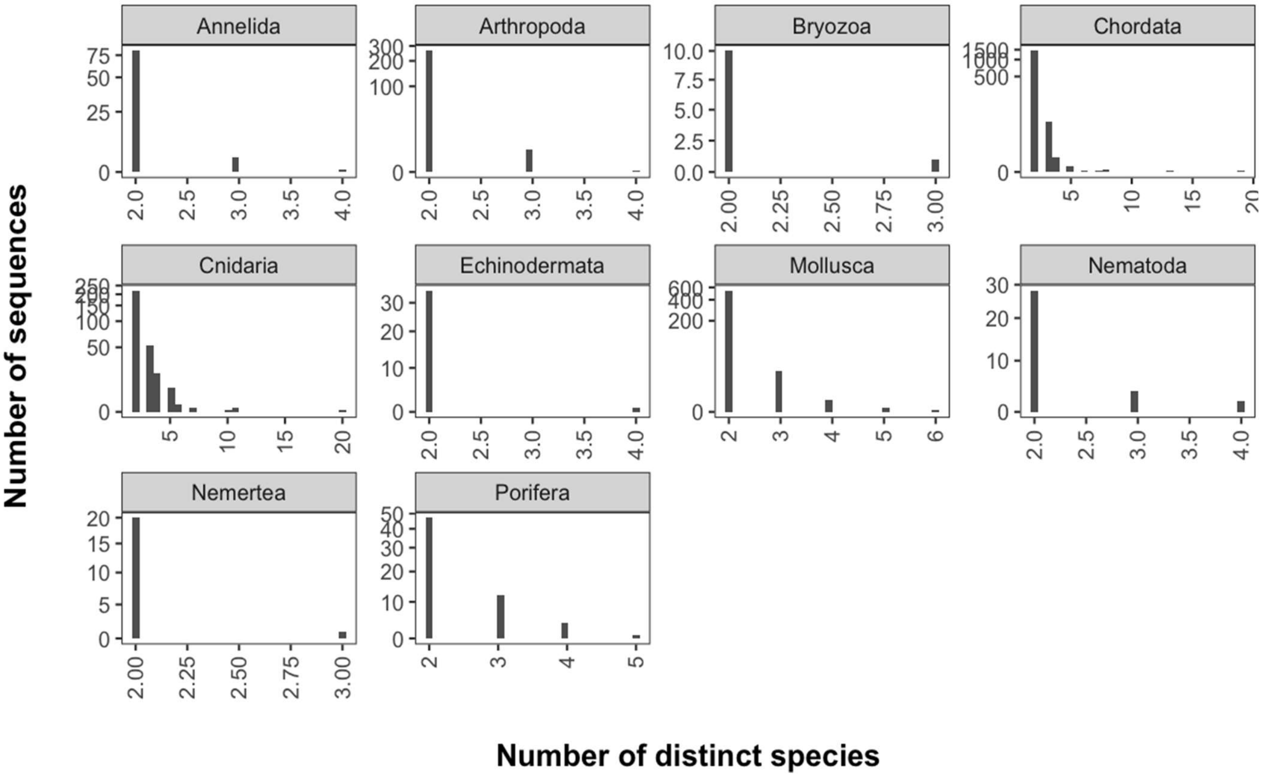
The issue is further complicated for speciose taxonomic groups. Some genera boast hundreds of species, and in most cases only a very small fraction of these will be represented in DNA reference databases, meaning that the analyst must confront the specter of a vast amount of missing data for the target taxa of most interest. For our own work, to consider a species "barcoded" we generally aim for at least 3–5 reference sequences per species per geographic region, often translating to dozens of sequences for widespread species likely to exhibit substantial genetic variation. For confident bioinformatic assignment, the reference data would also require adequate representation of congeners, so that the algorithm has adequate information with which to discriminate among them.

Ideally, available reference data should be sufficient to demonstrate a "barcode gap," the term used to describe a pattern in which *intra*specific divergence is non-overlapping with *inter*specific divergence (Čandek & Kuntner, 2015; see Box 1). COI famously shows such gaps across many animal clades (Andújar et al., 2018), though the effect is lineage- and marker-dependent and should be empirically verified for the taxa at hand. In groups without a clear barcode gap, species-level assignment should be treated with additional caution.

In addition to assessing adequacy of taxon representation in the reference database, it is critically important to assess phylogenetic consistency: Does the assigned taxon fall within a well-supported monophyletic clade when the query sequence is placed within a reference tree, as opposed to simply being matched by pairwise sequence similarity? Phylogenetic placement explicitly leverages evolutionary context and often outperforms pure similarity methods when close matches are absent or sequences are divergent, though these methods carry higher computational costs (Munch et al., 2008). Minimally, a computationally faster neighbor-joining tree could be used to check species clusters based on sequence similarity; even in these cases, assignment uncertainties can often be rapidly identified (Box 1).

Uncertainties in taxonomic assignments typically exist behind a veil, especially for end-users not trained in the analysis of metabarcoding data. For such users, assignments are likely taken as a given, particularly when those assignments are backed by statistics such as a very high degree of identity between the query and the reference sequence. What those users may not realize is that standard bioinformatic assignment methods may deliver a single species name even if there are multiple species with similarly strong matches to the query. When multiple species share identical sequences for the target marker, the final assignment obviously cannot be trusted; this problem occurs in many taxonomic groups (e.g., cnidarians, Fig. 2) and will often be exacerbated in metabarcoding studies due to reduced amplicon size. Such uncertainties are not always either explicitly handled or made transparent in standard bioinformatic workflows.

Figure 2. Number of distinct metazoan species with identical sequences for the COI marker. We used the MIDORI unique SP GB268 reference database, filtered out terrestrial and nonmetazoan taxa, removed hybrids, and kept only sequences with a binomial name and bp between 400 and 800 with equal or fewer than three ambiguities. We counted how many different species were associated with an identical sequence after standardizing species names to GBIF taxonomic backbone to remove cases where different species names were due to synonyms and outdated taxonomy. We used a pseudo_log_trans(10) scale to aid visualization, please note scales differ across panels. To simplify the figure, we did not include the phyla with only up to two different species per identical sequence (Acanthocephala: 1 seq; Brachiopoda: 1 seq; Chaetognatha: 5 seqs; Onychophora: 9 seqs; Platyhelminthes: 37 seqs; Rotifera: 5 seqs; Sipuncula: 1 seq). Although most shared sequences occur across different species in the same genus, many occur across different genera, families, orders, or even classes. For example, among the chordates, there were 20 instances of sequences shared between species in different classes, 59 shared between orders, 102 between families, and 346 shared between different genera.

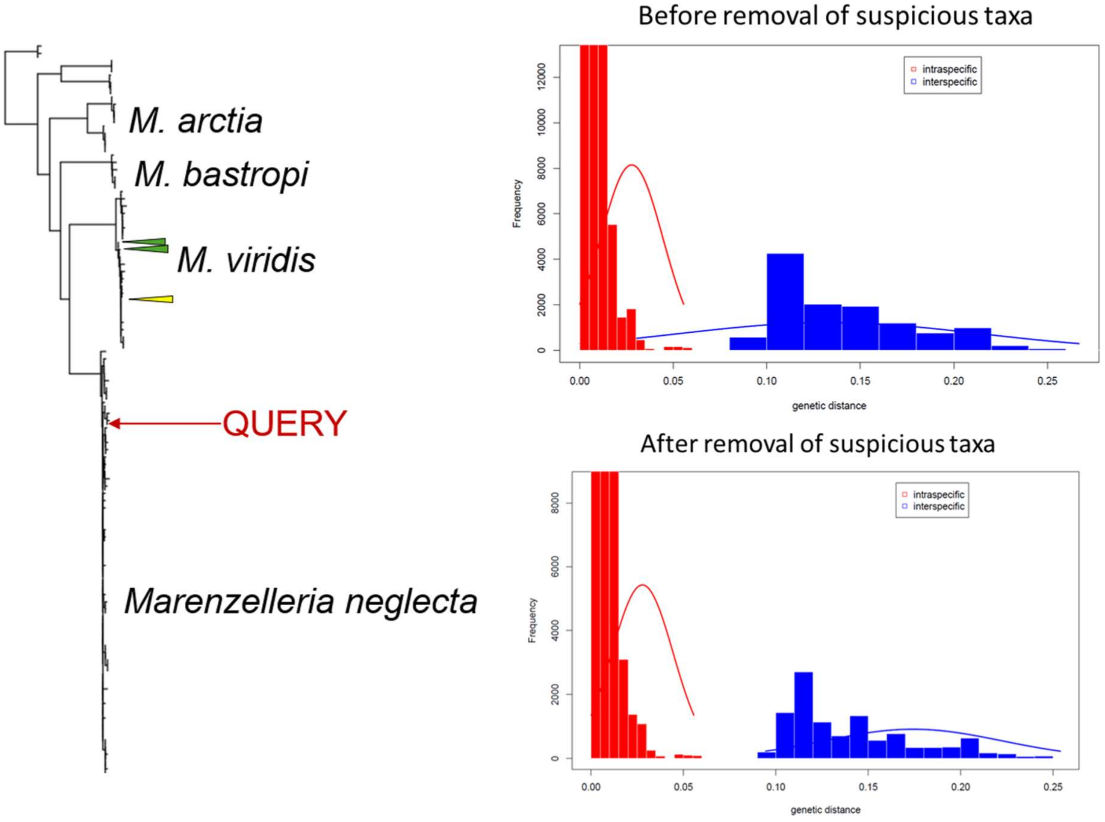


Box 1. Examples of tree-building to assess confidence in taxonomic assignment

Tree-building is a critically important exercise for assessing confidence in taxonomic assignments made by bioinformatics pipelines. Here, we showcase two examples of potential NIS identified by Pappalardo et al. (2025) in zooplankton samples from Prince Williams Sound, Alaska. For simplicity, the trees below were drawn using a Neighbor Joining algorithm implemented in the "Distance Tree of Results" option in the NCBI online BLAST engine. The first 100 hits returned by a blastn search for the query sequence were used for the analysis. We generated barcode gap plots on the same data using the *barcoding.gap* function of the R package *BarcodingR* (Zhang et al., 2020).

The first example, in which the query was initially assigned to the invasive spionid polychaete *Marenzelleria neglecta*, illustrates a best-case scenario for establishing confidence in a species detection. The ASV falls within a monophyletic clade of sequences from the same species, and congeneric sequences also fall within separate clades as outgroups to the *M. neglecta* clade. There are only 5 accepted species in this genus, and the first 100 hits include three of them, in addition to the target sequence, suggesting good coverage in the reference database.

Left: NJ tree based on top 100 hits for query identified as *Marenzelleria neglecta*. The query sequence is identified within a large clade comprising exclusively *M. neglecta* sequences. Within the *M. viridis* clade, green arrow heads indicate two sequences assigned to *Laonice cirrata*; the yellow arrowhead indicates a sequence assigned only at the family level to Spionidae sp. **Right:** Barcode gap analysis, with intraspecific distance shown in red and interspecific in blue. Top panel shows full dataset, bottom shows dataset after removal of suspicious taxa indicated in the NJ tree. Note that some interspecific comparisons on the left panel are hidden behind the bars showing intraspecific distance.

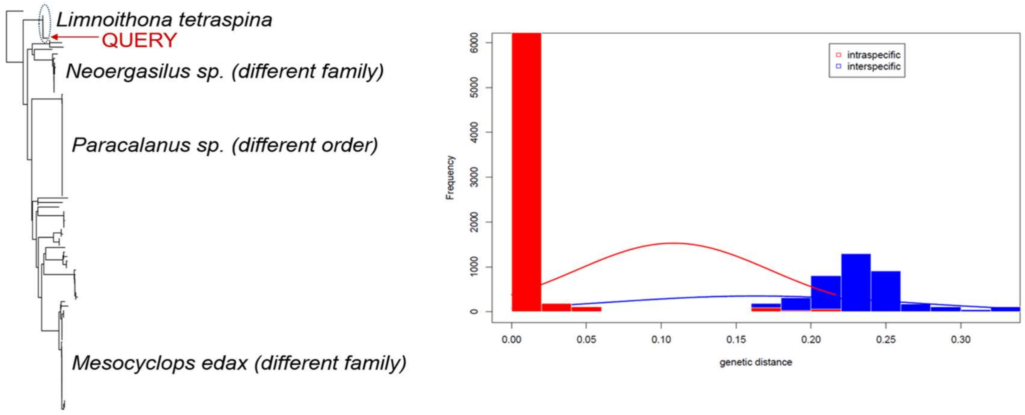


(Box 1, continued)

A barcode gap analysis shows a largely non-overlapping distribution, with a sharp peak of intraspecific distances and a much broader distribution of interspecific distances. Interestingly, that broad distribution draws attention to problems that may arise even in such “best-case” scenarios. Included in a large clade comprising sequences assigned almost exclusively to the congener *M. viridis* are one sequence assigned only at the family level (Spionidae sp.) and several assigned to the species *Laonice cirrata*. The negligible distance between these sequences and the *M. viridis* sequences result in deflated estimates of interspecific distance that skew that distribution to the left; if those sequences are removed from the analysis, the barcode gap becomes absolute. Although it is impossible to tell without further investigation, this observation raises the possibility that the *Laonice cirrata* sequences deposited in GenBank may be erroneously assigned to that species and actually represent sequences from *M. viridis*. Such errors are not uncommon and can significantly impact confidence in assignment in some cases.

The case of the cyclopoid copepod *Limnoithona* presents a quite different story. Initially, it is clear why the bioinformatic algorithm has made the assignment. The query sequence matches most closely with a sequence identified in the reference data as *L. tetraspina* and falls in a well-defined cluster that includes five other sequences also from that species. However, looking outside that cluster the resolution of the tree rapidly deteriorates. The most closely related sequence outside the *L. tetraspina* cluster, *Paracalanus aculeatus*, isn’t even in the same evolutionary order as the target species, and the rest of the tree presents a jumble of taxa in a pattern that fails to capture any evolutionary relationship between them. On top of this, of the species most closely related to *L. tetraspina*, none are represented in the tree; there is only one other known species in the genus, but there are also no representatives from the family Cyclopettidae to which the target belongs. There is, in fact, insufficient reference data to claim confidence in our species-level assignment, and the ability of the marker to resolve species-level differences in this group is unknown. Not surprisingly, this is reflected in the barcode gap, which in this analysis is virtually non-existent. All this is not to say that the assignment is *wrong*, only that we cannot confidently say it is *right*, and we should only be reporting it as a detection with appropriate caveats.

Left: NJ tree based on top 100 hits for query identified as *Limnoithona tetraspina*. The query sequence is identified, within a small clade comprising exclusively *L. tetraspina* sequences. No other confamilial species are represented in the tree. **Right:** Barcode gap analysis of *Limnoithona tetraspina* and related sequences. Note the complete overlap of intra- and interspecific distance distributions (red and blue lines).



More commonly, what may seem at first a reasonable assignment might become questionable when viewed in the context of existing reference data. The example of *Limnoithona tetraspina* shown in Box 1 provides a particularly clear example. An assignment that seems quite defensible at first glance turns out to be suspect on more thorough investigation, eroding confidence in the detection to the extent that reporting the presence of the species–even in the face of a strong detection signal–may be problematic without flagging the low confidence in the assignment. Multi-marker approaches offer a particularly valuable way to elevate confidence in detections despite uncertainties in taxonomic assignment, because independent primer sets targeting different gene regions are subject to different amplification biases, sequencing errors, and reference database artifacts—making it highly unlikely that the same spurious signal would appear in both. For example, Wu et al. (2023) designed and validated 138 primer sets across two gene regions (COI and cytochrome *b*) for 69 target invasive fish and aquatic invertebrates and demonstrated that read counts were significantly correlated with spiked DNA quantity when results were averaged across primers. More tellingly, their data show the power of cross-primer confirmation: species detected by only one primer with very low read counts—such as *Mnemiopsis leidyi*, which yielded 9 reads from one primer pair and zero from the other at the lowest spiking concentration—merited much less confidence than species detected robustly and consistently across both primer sets, such as *Salmo salar* (157 and 208 reads from the two markers, respectively) (Wu et al., 2023). The distinction between these cases rests not primarily on read count magnitude but on whether the detection replicates across independent analytical approaches.

Because bioinformatic choices can alter taxonomic calls (Ammon et al., 2018), assignments should ideally be tested for robustness across alternative pipelines and classifiers. Likewise, conclusions should be robust to reference database changes: curated, region-specific libraries and cleaned reference sets reduce false positives (Pappalardo et al., 2021) and can alter community composition estimates relative to uncurated GenBank pulls (Westfall et al., 2020). Ideally, the taxonomic assignments result from the comparison of multiple databases, as there are no perfect overlaps in the sequences deposited across reference databases. Finally, marker choice has implications that extend beyond the multi-marker replication discussed above. Because markers differ in taxonomic resolution and reference database coverage, species-level identifications should be evaluated in the context of marker-specific limitations. Genetic markers developed for specific taxonomic groups can also help to identify NIS (Westfall et al., 2020). Together, these practices—well-covered curated references, phylogenetic validation, evidence of a barcode gap, and robustness across pipelines, databases, and loci—provide a transparent framework for expressing and defending confidence in taxonomic assignment in eDNA metabarcoding studies.

3. Applying the validation criteria in practice

We recognize that application of these approaches represents a substantial additional burden on those looking to utilize metabarcoding data. Some users may not have the experience or resources to conduct all the analyses suggested above, and some assessments (such as establishment of ecological plausibility) require additional data or expert opinion that may not be readily available. We therefore do not suggest that all criteria must be thoroughly evaluated before metabarcoding results can be published or communicated to decision-makers or other stakeholders.

However, we suggest that each of the considerations described above *could* be addressed to more explicitly assess confidence that a novel detection of a non-native species is real and not an artifact of the method. Indeed, we think that evaluation of the validation criteria is likely feasible to at least some degree, even if only qualitatively, in most cases. Further, in situations where reporting of NIS detections might trigger management action, users should pursue these assessments to the degree possible. Most importantly, we think that awareness of these issues is paramount for both producers and users of metabarcoding data. The uncertainties that exist in detections, particularly in cases involving NIS or other species of concern, should be clearly recognized and made as transparent as possible, either through direct consideration of the issues described here or through open acknowledgment of uncertainties when they have *not* been addressed. We feel that such deliberation is particularly important for practitioners publishing results that highlight NIS observations in metabarcoding studies, even if those studies are conducted in a research setting (Darling et al., 2020).

We have not offered a full implementation of the analyses suggested, as that is beyond the scope of this perspective. It is likely that much of the above can be automated in ways that make it amenable to incorporation into analytical workflows applied downstream of standard bioinformatics pipelines. However, it is highly improbable that human intervention in the process can be completely avoided. For instance, even in very clear cases of confident taxonomic assignment there are often curious observations that require expert judgment (see Box 1, Panel 1). Expertise is likely to be even more important in assessing ecological plausibility. While existing data on environmental tolerances and species distributions are widely available, such data is often fragmented and frequently lags behind existing expert knowledge, particularly when it comes to NIS (Brock & Daehler, 2025). Interpreting the likelihood of pathway and vector connectivity represents an even greater challenge, and is often unclear even for the most well-studied NIS (Bailey et al., 2020).

Importantly, even if a researcher completes all verification steps and attests to a high level of confidence in the detection, this does not constitute a full risk assessment. That task remains with managers, informed by current evidence regarding the potential impacts of NIS and the necessity of mitigation. But the steps described here can help those managers prioritize from what can be bewildering lists of species names in metabarcoding outputs—a crucially important task when placing such outputs into decision-making contexts, essentially bridging the gap from data to actionable insights.

4. Conclusions

Metabarcoding has the potential to be an incredibly powerful tool for detecting NIS, particularly for early detection when preventing establishment is still possible. The genuine ability of eDNA metabarcoding to detect new introductions (Jeunen et al., 2022; Pearman et al., 2021; Van Nynatten et al., 2023) is one of the most compelling arguments for its adoption in biosurveillance programs. The goal of the framework described here is not to create additional barriers to the reporting of NIS detections, but to 1) help cut through the complexity of metabarcoding datasets to identify those detections in which confidence is highest, and 2) prevent disclosures of uncertain detections without appropriate caveats, which would potentially expose end-users to unnecessary risks. Ultimately, we believe this will enable both scientists and managers to focus attention and resources where they are most warranted.

The approaches suggested here can be adopted alongside multiple available tools developed elsewhere (e.g., replicated sampling designs, occupancy models, curated reference databases, minimum information reporting standards, and others) to improve the overall rigor and transparency of NIS surveillance using metabarcoding. The validation criteria described above may not be the only considerations needed to establish confidence in NIS detections,but represent critical minimal requirements that are amenable to analysis, at least in some form, by most practitioners. In addition to developing automated validation methods, future efforts may establish evidence-based confidence thresholds tailored to specific taxa, habitats, and risk assessment contexts, which will further increase confidence in detection and enhance the value of metabarcoding methods for NIS surveillance. In the meantime, we trust that the framework established here can help empower data producers and users to more confidently translate metabarcoding datasets into actionable insights for NIS management.

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