Eurasia Specialized Veterinary Publication

Entomological Research Letters

ISSN: 3108-639X

2024, Volume 4, Issue 1, Page No: 136-145
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Available online at: www.esvpub.com/

Molecular Barcode Partitions Become Biological Species Hypotheses Only After Explicit Translation Through Independent Taxonomic Evidence

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ABSTRACT

DNA barcoding has transformed insect discovery and identification by allowing sequence similarity, distance thresholds, and single-locus partition algorithms to organize large specimen sets into operational molecular units. The inferential problem begins when those units are treated as species without an explicit transition from molecular pattern to biological-taxonomic hypothesis. This article develops a taxonomic methodology in which barcode partitions are treated first as discovery units and only subsequently as species hypotheses when independently testable evidence supports that translation. The methodology distinguishes barcode clustering from the processes that may generate it, including sampling structure, geographically restricted lineages, introgression, deep intraspecific mitochondrial divergence, and analytical model behavior. Morphology is treated neither as an automatic veto nor as a privileged authority: it may corroborate molecular structure, reveal continuity, or reject apparent barcode subdivisions. Ecological and geographic evidence is similarly conditional, because host association, isolation, range discontinuity, and contact-zone behavior differ in evidential meaning. The resulting approach emphasizes evidence independence, explicit alternative explanations, taxonomic context, and traceable vouchers. It is intended to reduce two symmetrical errors: converting analytical partitions into species too rapidly, and dismissing biologically meaningful cryptic diversity because one conventional evidence class is uninformative. The proposed methodology does not provide a universal species threshold or scoring rule; it defines an auditable translation problem that can be resolved differently across taxa as evidence accumulates.

Keywords: DNA barcoding, Molecular operational taxonomic units, Species delimitation, Integrative taxonomy, Mitochondrial discordance, Insect systematics

Received: 24 May 2024
Revised: 22 June 2024
Accepted: 27 June 2024

How to Cite This Article: Hassan A, Siddiqui N, Khan B, Malik S, Ali H. Molecular Barcode Partitions Become Biological Species Hypotheses Only After Explicit Translation Through Independent Taxonomic Evidence. Entomol Res Lett. 2024;4(1):136-45. https://doi.org/10.51847/LGOrJzZOSF

Introduction

DNA barcoding is now embedded in insect discovery, monitoring, specimen association, and identification, but its operational success creates a taxonomic inference problem. Large-scale workflows can use mitochondrial sequences to sort specimens rapidly into molecular operational taxonomic units (MOTUs), after which independent evidence may be brought to bear on their biological meaning [1]. The methodological weakness

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arises when this sequence of operations is compressed and a molecular partition is treated as if the partitioning process had already established the existence, limits, and nomenclatural status of a biological species.

That shortcut is difficult to justify across insects because barcode divergence is not governed by a universal relation between sequence distance and species boundaries. An insect-wide analysis of COI variation showed that unusually high intraspecific divergence can generate false-positive splitting under threshold-based delimitation [2]. In green lacewings, threshold performance changed with sampling completeness, representation of close relatives, and analytical treatment [3]. In Entiminae weevils, the distribution of intra- and interspecific distances did not support a single subfamily-wide barcode gap, while genus-level behavior differed [4]. These findings do not negate barcode utility; they show that a partition is partly conditional on the taxon, reference set, and analytical rule that produced it.

The same caution applies when molecular assignments appear concordant with morphology. Comparisons among standard barcoding, metabarcoding, and morphology show that agreement varies with taxon, geographic context, threshold choice, and the identification task itself [5]. Identification concordance therefore cannot be converted directly into evidence that an inferred molecular boundary is a species boundary. This article addresses that gap by proposing an explicit translation step between a barcode-derived discovery unit and a biological species hypothesis. The proposed methodology asks what produced the partition, which independent evidence can genuinely contradict it, what alternative explanations remain viable, and what taxonomic claim is justified at the present evidential state.

Barcode partitions as discovery units

The most defensible initial role of a barcode partition is operational rather than ontological. Dense COI reference libraries can identify many insect taxa efficiently while still containing taxon-specific failures. In Dacini fruit flies, a large reference set demonstrated substantial identification utility, but simple distance criteria and monophyly were not uniformly concordant across all sampled species [6]. Such exceptions matter because a unit recovered by a barcode rule is an analytical output: it may correspond to a recognized species, a population lineage, a hybridizing complex, an overlooked species, or a cluster generated by sampling and model behavior.

Discovery units are nevertheless biologically valuable even before their species status is resolved. In paedomorphic Lycidae from the Puerto Rican Bank, DNA barcodes linked highly dissimilar life stages and sexes that conventional morphology alone could not readily associate [7]. This is an important distinction: the barcode can establish specimen association or reveal a recurrent lineage pattern without simultaneously proving that every distinct lineage represents a species. The value of the molecular unit lies in making a tractable target for subsequent taxonomic testing.

That testing also depends on traceability. Large barcode infrastructures can connect sequences to vouchers, images, collection metadata, and retained DNA, allowing later investigators to re-examine the physical basis of a partition [8]. Such linkage is especially important when operational units are generated automatically, because algorithms do not discover species in an assumption-free way. ASAP, for example, ranks alternative partitions from single-locus distance information [9], whereas mPTP infers putative units from branching patterns and rate processes on a single-locus gene tree [10]. Both can be useful discovery tools, but neither removes the inferential boundary between sequence partition and organismal species.

Accordingly, this article uses “barcode partition” for a reproducible grouping generated primarily from a barcode locus and “discovery unit” for the provisional taxonomic object that grouping creates. A discovery unit becomes more than a sorting device only when evidence external to the original partitioning process is used to test competing biological explanations. This distinction is proposed here as a procedural safeguard, not as a new species concept.

Sources of barcode clustering

Barcode clusters can arise for several biologically different reasons, and those reasons have different consequences for taxonomy. Deep mitochondrial divergence within a species can mimic hidden species, whereas mitochondrial sharing among otherwise differentiated taxa can erase a true species boundary. In caddisflies, ddRAD data showed that unusual mtDNA patterns, including low interspecific and high intraspecific divergence, did not map simply onto nuclear differentiation [11]. The relevant lesson is not that mitochondrial evidence is inferior, but that mitochondrial history is only one component of lineage history.

Geographic history is a second source of structure. Comparative phylogeographic work in widely distributed arthropods demonstrates that isolation, historical connectivity, and regional persistence can generate strongly structured mitochondrial lineages without making taxonomic rank self-evident [12]. In insects, this difficulty becomes acute when populations are allopatric and no direct transition zone can be sampled. A deep barcode split may then be compatible with prolonged population isolation, incipient lineage divergence, or independently evolving species, and the barcode alone cannot choose among these alternatives.

Introgression creates the opposite problem. In the *Bactrocera dorsalis* complex, population-scale RAD-seq recovered genomic species structure that COI failed to diagnose reliably for several taxa because mitochondrial introgression crossed species boundaries [13]. Thus, absence of a barcode gap cannot be treated as evidence that independently differentiated species do not exist. Conversely, nuclear genomics does not create an automatic species verdict. Comparative work across highly allopatric Lepidoptera showed heterogeneous correspondence between mitochondrial differentiation and nuclear genomic structure [14], emphasizing that genomic corroboration must be interpreted in the geographic and taxonomic context in which it was generated.

This matters because multispecies-coalescent methods can themselves convert geographic population structure into excessive species partitions. Analyses of geographically widespread taxa have demonstrated over-splitting under some multispecies-coalescent applications [15], while simulation work has shown more generally that such models can delimit population structure that need not be equivalent to completed species boundaries [16]. More recent migration-aware approaches explicitly model gene flow and can reduce some forms of over-splitting, but their outputs remain dependent on assumptions, parameterization, and the biological scenario under consideration [17]. Genomic evidence is therefore potentially powerful corroboration, but it should not replace "barcode authority" with "genomic authority."

The practical implication is that the first taxonomic question should not be "How divergent is this barcode cluster?" but "What processes could plausibly have produced this partition?" Deep within-lineage divergence, incomplete geographic sampling, allopatry, introgression, mitochondrial capture, population structure, and algorithmic sensitivity have different empirical signatures and require different tests. The proposed methodology therefore treats a barcode partition as an inference-generating observation whose biological interpretation remains open until competing causes have been evaluated.

Independent morphological testing

Morphology is most informative when it is used as an independent test rather than as a retrospective description of molecular groups. Integrative work on *Grebennikovius* dung beetles shows that geographically localized molecular lineages can be supported by independently observed morphological differentiation [18]. Conversely, studies of Italian *Meloe* demonstrate that substantial lineage divergence may coexist with morphological similarity, so absence of conspicuous external differences cannot function as an automatic veto against cryptic species hypotheses [19].

The taxonomic importance of morphology also extends beyond phenotypic corroboration. Revisionary work on *Cerurina* and *Afrocerura* integrates molecular evidence with historical specimens and type-based morphology, illustrating that sequence data do not displace the nomenclatural and comparative functions of museum material [20]. In *Nylanderia* ants, male genital morphometrics provided an especially strong independent test: phylogenomic and morphological evidence supported some species-level distinctions while three previously reported COI clades within *N. fulva* were not supported by those independent data [21]. The same evidential direction does not always occur. Genomic work on the *Melanoplus scudderi* complex recovered relationships consistent with morphologically recognized species despite earlier molecular paraphyly [22].

These cases argue against any fixed hierarchy in which morphology is either sovereign or obsolete. Morphological evidence can corroborate, contradict, or remain uninformative, and its strength depends on the character system, sex, life stage, specimen quality, and evolutionary depth being examined. Continuous variation adds another difficulty. In *Carabus nankotaizanus*, morphological and geographic patterns required integrative interpretation rather than simple conversion of discontinuities into species boundaries [23]. In the proposed methodology, morphology therefore functions as a falsifiable evidence stream: it is valuable precisely because it can disagree with the barcode partition.

Ecological and geographic evidence

Geographic separation is frequently associated with barcode divergence, but separation itself is not a species criterion. When spatially differentiated populations meet, contact-zone structure can help distinguish ordinary geographic variation from more persistent lineage boundaries; where contact is not sampled or does not exist, the inference is necessarily weaker and alternative explanations remain more difficult to eliminate [24]. This makes geography an evidential modifier rather than a direct translation rule.

Genome-wide phylogeographic structure can strengthen a lineage hypothesis when multiple analyses recover geographically coherent units. Asian cavity-nesting honeybees provide an example in which broad geographic sampling and genome-wide SNP analyses revealed strongly structured lineages [25]. Even here, the evidential meaning comes from the conjunction of geographic pattern and independent genomic structure, not from distance between localities alone. Similar logic applies to ecological association. In *Pseudoloxops* plant bugs, initial molecular groupings were explicitly tested using morphology, genetic evidence, host association, and geography [26]. Host use and island distribution therefore become informative because they test whether the molecular partition predicts independently observed biological organization.

Negative ecological or morphological evidence should be interpreted asymmetrically. A lineage that lacks obvious morphological differentiation is not necessarily taxonomically empty: a systematic assessment of multilocus insect studies found substantial morphologically cryptic diversity across the literature [27]. Yet ecological or geographic association can also arise within species, particularly when host use, habitat, or dispersal limitation produces population structure. The taxonomic question is therefore not whether a barcode cluster has an associated habitat, host, or range, but whether those associations are repeatable, independently measured, and difficult to explain under plausible within-species structure.

For this reason, ecological and geographic information is most useful when it changes the set of viable explanations rather than merely adding another descriptor to a molecular cluster. The matrix below distinguishes the principal contexts in which these data strengthen, weaken, or leave unresolved the translation of a barcode partition.

Table 1. Ecological and geographic conditions that alter the interpretation of barcode-derived discovery units

Spatial opportunity and contact		
Ecological/geographic condition	**Unsampled allopatry**	**Sampled contact zone**
Observed partition evidence	Barcode divergence plus geographic separation	Spatial transition in molecular, morphological, or ecological traits
Effect on interpretation	Partition remains compatible with species divergence or structured populations	Can distinguish persistent lineage separation from clinal within-species variation
Taxonomic setting	Spatially separated populations with no observed contact	Divergent units approach or overlap geographically
Next test	Nuclear genomic comparison and denser geographic sampling	Genotype–geography and phenotype transition analysis
Remaining alternative	No direct evidence of transition, gene flow, or reproductive interaction	Contact may be recent, narrow, or environmentally structured
Standing	Evidence-bounded interpretation	Evidence-bounded interpretation

Geographic or ecological concordance			
Ecological/geographic condition	**Geographic–genomic concordance**	**Host-associated partition**	**Morphologically cryptic geographic lineage**
Observed partition evidence	Concordant population-genomic structure across geography	Repeatable host association measured independently of barcode assignment	Molecular and geographic coherence with weak external differentiation
Effect on interpretation	Strengthens the biological plausibility of the partition	Adds ecological differentiation to the hypothesis	Absence of obvious morphology should not automatically collapse the partition

Taxonomic setting	Spatially coherent units also recovered by independent nuclear data	Barcode units differ in host use or resource association	Spatially structured molecular lineage lacks obvious diagnostic morphology
Next test	Tests of migration, continuity, and independent phenotype	Replication across sites, life stages, and genomic backgrounds	Targeted morphology plus independent nuclear data
Remaining alternative	Population structure can still be overinterpreted as species structure	Host races, plasticity, or sampling bias can mimic species-level association	Cryptic diversity and population structure remain competing explanations
Standing	Evidence-bounded interpretation	Evidence-bounded interpretation	Evidence-bounded interpretation

Evidential reduction	
Ecological/geographic condition	**Proposed evidential-reduction state**
Observed partition evidence	Initial partition loses host, range, or locality specificity
Effect on interpretation	Weakens the case that the barcode boundary tracks an independently organized lineage
Taxonomic setting	Ecological/geographic associations disappear under broader sampling
Next test	Re-estimate partition and test alternative population models
Remaining alternative	Broader sampling may reveal newly structured subregions
Standing	Proposed synthesis

Reproductive and genomic corroboration

Independent genomic evidence is most informative when it tests a mitochondrial partition rather than merely redescribing it with more markers. In *Aeschyntelus*, morphology, mitochondrial sequences, and nuclear SNP data exposed mitonuclear discordance, demonstrating that different genomic compartments can retain different histories [28]. Such disagreement does not identify its own cause: incomplete lineage sorting, introgression, demographic history, and analytical limitations remain alternative explanations unless independently tested.

Genomic evidence can also reverse an apparent taxonomic split. In the *Bactrocera frauenfeldi* complex, hundreds of nuclear loci, morphology, and biogeography supported synonymy of a variable morphotype rather than recognition of an additional species [29]. Thus, integrative evidence is intrinsically bidirectional: it may strengthen, divide, merge, or leave unresolved an initial molecular partition.

Reproductive evidence requires similar restraint. Species-delimitation models can accommodate nonzero gene flow, showing that migration need not automatically imply conspecificity [30]. Conversely, detecting genomic differentiation does not establish complete reproductive isolation. Migration-aware coalescent analyses further show that explicitly modelling gene flow can change inferred boundaries [17]. The relevant question is therefore not whether reproductive or genomic evidence reaches an absolute threshold, but whether it removes credible alternatives to interpreting a barcode partition as an independently evolving biological lineage.

These distinctions require a visual separation between mitochondrial observation, independent genomic or reproductive tests, and the taxonomic inference drawn from their agreement or conflict. The resulting architecture is summarized in **Figure 1**.

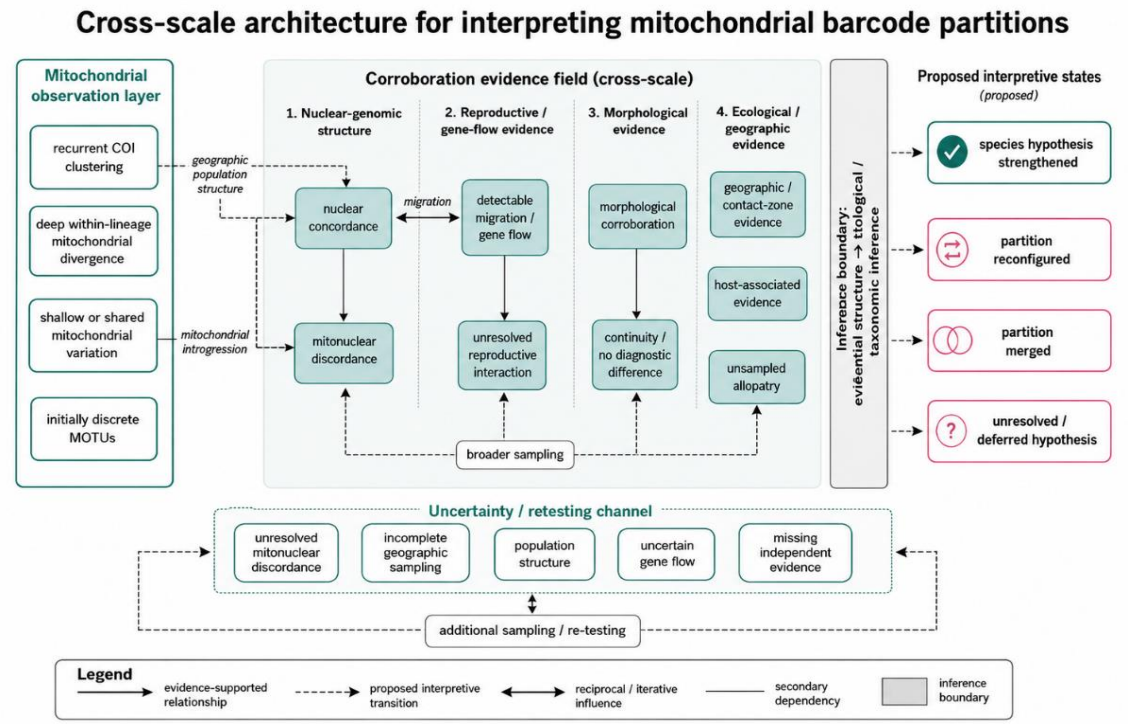


Figure 1. Independent genomic and reproductive tests expose where barcode partitions gain, lose, or retain taxonomic ambiguity

Translation from motu to species hypothesis

The transition from MOTU to species hypothesis should begin by recognizing that repeated recovery of a partition is not necessarily independent corroboration. Simulations comparing single-locus methods show that algorithms differ in their tendencies to over-split or over-lump under different conditions [31]. Agreement among algorithms operating on the same locus may therefore indicate analytical robustness without demonstrating biological independence. ASAP partitions remain products of distance structure [9], whereas mPTP partitions remain products of a gene-tree model [10].

The central contribution of this article is a proposed translation rule: a barcode-derived MOTU should be treated initially as a discovery partition, then promoted to a biological species hypothesis only when evidence not logically generated by the original barcode partition reduces credible alternative explanations. Independence is therefore functional, not merely technical. Nuclear loci, morphology, host association, contact-zone behavior, geography, or reproductive information contribute most when they could realistically contradict the barcode result.

No universal evidence hierarchy follows from this rule. Nuclear structure can itself reflect population subdivision, allopatric divergence can remain ambiguous, morphology can be absent despite meaningful lineage separation, and gene flow can persist across taxonomic boundaries. Translation must therefore remain defeasible: strong independent concordance can strengthen a species hypothesis, discordance can reconfigure it, contradictory evidence can collapse it, and insufficient evidence can justify deferral rather than forced naming.

The translation states and their associated tests are condensed in **Table 2** because their value lies in distinguishing logically different inference outcomes rather than assigning numerical confidence.

Table 2. Translation states governing whether a barcode-derived MOTU remains provisional, strengthens, or changes taxonomic interpretation

Starting partition		Change in taxonomic force		What resolves the state	
Translation state	**Independent evidential condition**	**Taxonomic move**	**Competing explanation**	**Next test**	**Proposed standing**
Provisional discovery units					
Barcode-only discovery unit	Partition recovered from one mitochondrial	Retain as provisional discovery unit	Sampling structure, deep intraspecific divergence,	Independent nuclear, morphological,	Proposed rule

	locus or closely dependent algorithms		analytical partitioning	ecological, or geographic test	
Repeated but non-independent molecular partition	Similar unit recovered by multiple analyses of the same underlying barcode signal	Analytical robustness increases, but biological status remains unresolved	Shared-data dependence	Test an evidence class capable of contradicting the partition	Proposed rule
Corroboration and discordance					
Independently corroborated partition	Barcode boundary aligns with separately measured genomic, morphological, ecological, geographic, or reproductive organization	Strengthen as biological species hypothesis	Parallel population structure or shared environmental covariation	Target alternative explanations directly	Proposed rule
Mitonuclear or cross-evidence discordance	Mitochondrial partition conflicts with nuclear, morphological, or geographic evidence	Reconfigure or defer the hypothesis	Introgression, incomplete lineage sorting, population structure, sampling bias	Broader nuclear sampling, contact-zone analysis, targeted morphology	Proposed rule
Resolved or unresolved translation					
Evidence-supported merger	Independent evidence fails to maintain an apparent barcode or morphotype boundary	Collapse or synonymize the candidate partition where nomenclaturally justified	Recent divergence or insufficient sampling	Expanded geographic and genomic sampling	Evidence-bounded outcome
Unresolved translation	Available evidence is sparse, non-independent, or mutually contradictory	Do not force species recognition or rejection	Multiple viable biological histories	Acquire the evidence class with greatest discriminating power	Proposed rule

Decision outcomes

The proposed methodology deliberately permits more than a binary "species/not species" outcome. Independent concordance can strengthen a barcode-derived species hypothesis, as illustrated when morphology and phylogenomics recover the same taxonomic distinctions [21]. That outcome should still be described as support rather than universal confirmation because evidence classes may share hidden geographic or sampling structure.

The opposite outcome is equally important. Independent evidence can merge candidate units, as occurred when phylogenomics, morphology, and biogeography supported synonymy within a *Bactrocera* complex [29]. Discordance can also justify deferral. Introgressed mitochondrial histories [13], strongly allopatric sampling [14], migration [17], or incomplete contact-zone evidence [24] may prevent a decisive interpretation even when molecular structure is conspicuous.

Accordingly, the proposed decision vocabulary is deliberately asymmetric: retain as discovery unit, strengthen as species hypothesis, reconfigure, merge, or defer. These are evidential states, not formal nomenclatural acts. Formal taxonomic change requires the separate diagnostic, nomenclatural, and documentation work appropriate to the focal group.

Reporting and voucher standards

A molecular partition can be reassessed only if its empirical basis remains recoverable. Voucher-linked barcode libraries demonstrate that sequence records can be connected to physical specimens, images, locality information, and retained DNA [8]. Such linkage should be preserved when a MOTU is translated into a species hypothesis so that later investigators can repeat identifications, inspect morphology, and test alternative explanations.

Taxonomic data remain unevenly preserved across repositories and publications, with substantial gaps in the mobilization of specimen, character, and other reusable evidence [32]. This article therefore proposes that reports distinguish explicitly between the partition-generating evidence and the independent translation evidence. At minimum, authors should state how the molecular partition was produced, which vouchers underlie it, what

geographic and life-stage sampling was available, which independent evidence classes were tested, which evidence conflicted, and which important tests remain absent.

This reporting architecture is not a new nomenclatural code. It is an auditability requirement for molecularly initiated taxonomy. Missing evidence should be recorded as missing rather than filled by inference, and unresolved discordance should remain visible so that future sampling can alter the hypothesis without reconstructing the original analytical chain.

Conclusion

Molecular barcode partitions are valuable because they make insect diversity searchable, comparable, and testable at scales that morphology alone often cannot achieve. Their taxonomic meaning, however, is not contained automatically within the partition itself. The methodology developed here separates molecular discovery from biological inference and requires explicit translation through evidence capable of contradicting the original barcode pattern. Morphology, geography, ecology, reproductive information, and genomics can each strengthen, weaken, merge, or leave unresolved a candidate lineage, and none functions as a universal authority. Treating species hypotheses as defeasible translations rather than direct outputs of clustering preserves the speed of molecular discovery while making the subsequent taxonomic reasoning more transparent, reproducible, and appropriately bounded.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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