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The Case for a Nuclear Barcode: Using the CAD CPS Region for Species and Genus Level Discrimination in Beetles

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Abstract: DNA barcoding has revolutionized how we discover, identify, and detect species. A substantial foundation has been established with millions of mitochondrial cytochrome c oxidase I sequences freely available for eukaryotes. However, issues with COI ranging from uniparental inheritance and small genetic population sizes to nuclear and asymmetric introgression can impede its use. We propose using CAD as the "nuclear barcode" to complement the COI barcode and ameliorate these concerns. We focused on beetles from taxonomically diverse species-level studies that used COI and CAD. An ambiguous barcode gap was present between intra- and interspecific genetic distances in CAD and COI; this led to difficulty with automated gap detection methods. We found pseudogenes, problematic population structure, introgression, and incomplete lineage sorting represented in the COI data. A CAD gene tree illuminated these cryptic problems. Placement tests of species and outgroups using distance-based tree building were largely successful for CAD, demonstrating its phylogenetic signal at the species and genus levels. Species placement issues were typically unique to one locus, allowing for recognition of misdiagnosis. We conclude that a CAD barcode is a valuable tool for beetle diagnostics, metabarcoding, and faunistic surveys.

Keywords: barcoding; eDNA; biodiversity; species discovery; identification; invasive species; integrative taxonomy

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1. Introduction

The use of DNA sequences for species identification has transformed taxonomy [1]. A relatively short DNA segment can provide additional characters and, sometimes, the only characters to diagnose closely related, morphologically similar species. As a result, the identification of morphologically ambiguous species, body fragments, feces, and trace cells is possible by matching a DNA sequence with a library of known sequences. DNA-based identification alleviates reliance on examining complete specimens, e.g., [2]. Taxonomically uneducated personnel can perform the technical aspects of generating DNA data followed by a taxonomic expert evaluating the results, increasing the rate and accuracy of specimen identification, e.g., [3]. In addition, DNA identification can provide initial evidence of cryptic or pseudo cryptic species, which may instigate a taxonomic revision and result in a better understanding of the taxon's diversity, e.g., [4,5].

To achieve a reliable and accurate DNA identification of animals, a database of DNA sequences representing intraspecific-level diversity is necessary because gaps in this database can result in misidentifications or erroneous declarations of new species, e.g., [6]. Several mitochondrial regions have been used at the species level; however, most have not achieved wide-scale use [7–10]. Currently, a segment of the mitochondrial cytochrome c oxidase I DNA sequence is promoted and used as the "barcode" for identifying animals [11]. More than 12 million sequences have been generated in the past 20 years (https://boldsystems.org, accessed on 23 January 2023) and have been included in more than 3700 "DNA barcoding" studies [12]. Although the utility of mtDNA COI sequences to

help resolve taxonomic mysteries is undeniable, reliance on one "barcode" gene has been discussed [13]. The unique properties of mtDNA genes make them subject to several issues that can lead to misidentifications. Most importantly, maternal inheritance of the mitochondrial genome may not reflect the evolutionary history of the species and thus not diagnose them [14]. Heteroplasmic and pseudogenic mtDNA (NUMTS) are non-homologous mitochondrial genes that can mislead diagnoses [15–17]. Variable nucleotide substitution rates among taxa make applying a standard barcoding gap difficult [18]. Thus, a gene with a different evolutionary history and inheritance pathway could augment or enhance established mtDNA barcoding identification.

Several nuclear genes have been implicitly and explicitly used for the DNA identification of plant and animal species, e.g., [19–21]. The internal transcribed spacer 2 and D1–D2 region of the nuclear ribosomal gene complex have been shown as potential universal barcoding loci due to conserved PCR primer sites, ease of amplification from specimens in a variety of conditions, and the ability to discriminate closely related species [19,21]. Potential issues that may hinder the use of ribosomal genes for identification include variable sequences within individuals due to a lack of concerted evolution among paralogous ribosomal copies and inconsistent alignment of length variable sequences due to the insertion/deletion of nucleotides, although the D1–D2 region demonstrates little intra-individual polymorphism [21–23]. Selecting exons of single or low-copy genes as a "barcode" gene can potentially circumvent the issues inherent in ribosomal genes. Nuclear barcodes had limited success in identifying closely related plant species. However, exon + intron gene sequences can discriminate specific plant taxa [24,25]. Among animals, these genes have been mainly used in phylogenetic reconstruction. Their targeted application for species identification has not been widely explored [26]. This is likely due to the inclusion of only a few individuals of a species in most studies; thus, intra- vs. interspecies sequence variation is not readily comparable [27].

In a few studies, partial CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase) nuclear gene sequences were useful in the identification of fly and beetle species [20,28,29]. Given the development and use of the CAD CPS (carbamoyl-phosphate synthetase 2) region in beetle phylogenetic studies [30–33], data exist to explore its use as a nuclear barcode gene for beetles. For example, Cognato et al. [20] created a barcoding database of partial CAD sequences for ambrosia beetles (Scolytinae: Xyleborini). Species were confidently identified when the intraspecific difference was <1%; differences above that intraspecific level increased the possibility of misidentification, and species were morphologically diagnosable when the interspecific sequence difference was >3%. This dataset complimented COI barcodes that exhibited uncommonly high intraspecific sequence differences (>10%) stemming from the high female-to-male ratio in these haplodiploid beetle species. Given this preliminary evidence, we investigated the potential of CAD as a nuclear barcoding gene for beetles. We assembled previously published COI and CAD sequences with sufficient intraspecific sampling for beetle families Cerambycidae [33], Curculionidae: Scolytinae [20,34–36], and Carabidae [31,37]. We analyzed these datasets by evaluating their composition, performing taxon placement tests, and measuring intra- and interspecific differences to assess the occurrence of barcoding gaps. We conclude that CAD is a functional barcode with some limitations, as with COI, and we promote its use to complement mitochondrial data in species diagnosis and delimitation.

2. Materials and Methods

2.1. Taxon and Gene Selection

We aimed to capture the breadth of diversity in *Coleoptera* when choosing specimens for this study. To this end, considering available datasets including both genes, we focused on the genetically diverse *Phytophaga* and extended to the *Carabidae* in the other major *Coleoptera* suborder, *Adephaga*. The weevil tribe *Scolytinae: Xyleborini* is known to have larger cytochrome c oxidase I distances between species due to their haplodiploid lifestyle [20]; thus, species in the genera *Xyleborus* and *Cyclorhipidion* were sampled. The

outbreeding scolytine weevil genus *Scolytus* was included as a relative of the *Xyleborini*. The *Cerambycidae* are rich in species and are represented by the well-sampled genus *Monochamus*. The final genus, *Bembidion*, is a large, well-studied group of ground beetles [31,37]. As taxon sampling within a genus depends on the original studies, some genera are more thoroughly sampled. *Bembidion* included multiple subgenera but low intraspecific sampling, *Monochamus* included 17 of 18 species in the clade with approx. four geographically diverse samples per species, *Scolytus* had 40 spp. CAD/34 spp. COI of ~127 spp., *Cyclorhipidion* 12 spp. CAD/17 spp. COI of 109 spp., and *Xyleborus* is restricted to a monophyletic clade of four species due to non-monophyly of the genus but also includes the most intraspecific sampling in our study. Species information, outgroup taxa, and GenBank numbers are available in Supplementary Table S1. All chosen genera provide intraspecific data for both genes for multiple species to allow for evaluation and comparison.

COI is the standard animal barcode. As a ~640 bp segment is the current full-length standard, we adjusted longer sequences to this length. Also, we included some shorter sequences down to 220 bp because a short COI fragment has been shown to function in diagnostics [11,38], but see Will and Rubinoff [39]. The PCR primers used to amplify this region for the included taxa varied (Table 1).

The carbamoyl-phosphate synthetase 2 locus of CAD has been useful in species-level phylogenetic studies [32,37,40] and species diagnostics [21] (Gorring unpub.). We evaluated the <1000 bp region sequenced for each genus and reduced the length of certain long *Bembidion* sequences to a shorter shared region. The length of the CAD region varied in the focal genera due to the development of multiple primers (Table 1). Though multiple primers have been developed, amplification is generally simple for short portions or with a standard nested PCR.

Several sequences of *Monochamus* and *Goes* were produced for this study using hybrid enrichment: GenBank COI: OR145287–OR145289, CAD: OR195144–OR195150. Briefly, DNA was extracted with a standard column and libraries were prepared and enriched for a set of genes by adapting PCR-based hybrid enrichment protocols developed in vertebrates [41,42] (de Medeiros et al. in prep). Illumina PE150 short read sequencing was performed and the resulting reads were mapped to congener sequences in Geneious Prime 2022.1.1 (available from https://www.geneious.com, accessed on 23 January 2023) for downstream analysis.

Most included sequences came from published studies, as cited above. For each genus, these sequences were downloaded from GenBank at https://www.ncbi.nlm.nih.gov/ (accessed 23 January 2023) using the GenBank IDs in Table S1. A small number of sequences that were produced in those projects are now submitted to GenBank: COI: OR145282–OR145286, OR145290–OR145291; CAD: OR195143. All sequences were aligned by subset using the MAFFT online server available at https://mafft.cbrc.jp/alignment/server/ (accessed 12 February 2023). If needed, the aligned file was manually corrected and trimmed in Mesquite v. 3.5 [43].

2.2. Gene Statistics

Summary statistics were calculated to compare basic measures of the genes. To determine the number and percentage of parsimony informative sites in each dataset, the R v. 3.5.1 package Phyloch v. 1.5-5 [44] was used strictly on the genera, without outgroups. All pairwise distances were calculated within each complete dataset using PAUP* v. 4.0a169 software [45]. The uncorrected pairwise distance was used, as it has been demonstrated to be most useful for closely related sequences [46]. The distances were then plotted in a box and whisker design to demonstrate the reality of barcode gaps for each group in the two genes.

To evaluate nucleotide saturation, we analyzed the third codon position of each dataset. Each aligned file was individually executed in PAUP*, 3rd positions were isolated for analysis, and the number of pairwise transitions and transversions was calculated. These values were then compared to the overall uncorrected p-distance.

2.3. Tests of Specimen Placement

Taxon placement tests were performed at two classification levels to determine if a neighbor-joining tree-building method could place the taxa appropriately (e.g., with a conspecific or outside as proper). For species-level determination, the percentage of individuals grouped with a conspecific in the NJ tree was calculated; if a tip was grouped with a conspecific or was a singleton, a score of one was given. Each taxon set was arranged to include four outgroups: one from a different subfamily, one from another tribe in the same subfamily, and two contribal genus outgroups with varied branch lengths if known. Outgroup placement was tested with a neighbor-joining tree in PAUP* using the other subfamily as the assigned outgroup, uncorrected p-distances, ties broken randomly, and default settings otherwise.

To enable a comparison with an automated method of species delimitation, we analyzed each dataset using assemble species by automatic partitioning, ASAP, which is an advancement in automatic barcode gap discovery [47,48]. The main advancement is giving a score to each hierarchically clustered partition estimated from pairwise distances. The ASAP score combines a *p*-value calculated through intra- vs. inter-grouping statistics and the relative size of the gap present based on a threshold value. The aligned sequence data for each set were input into the ASAP website (https://bioinfo.mnhn.fr/abi/public/asap/, accessed on 1 May 2023) for analysis using the p-distance option.

Finally, we tested the placement of new morphologically identified specimens within an NJ tree. This was built on the *Monochamus* COI and CAD datasets and the same tree-building parameters as above. This set of specimens was derived from intercepted specimens identified by national identifiers [49]. They consisted of geographically heterogeneous *Monochamus* species present in our base *Monochamus* dataset.

3. Results

3.1. Comparison of COI and CAD Gene Characteristics

The characterization of the traditional beetle "barcode" locus, mitochondrial COI, and nuclear CAD gives similar proportions of parsimony informative sites for the outbreeding genera *Monochamus* (0.18 COI/0.10 CAD), *Bembidion* (0.22/0.20), and *Scolytus* (0.41/0.33); the difference between the genes is larger in the haplodiploid genera *Xyleborus* (0.27/0.03) and *Cyclorhipidion* (0.37/0.12) (Table 1). At least 10% of the CAD sites are informative in the outbreeding genera, and sister species retain diagnostic sites. There are ~20 diagnostic bases in the sister pair *Monochamus sutor* and *M. galloprovincialis*, which only has one fixed difference and an indel in 28S [50]. Even in the narrowly taxon-sampled *Xyleborus*, with 3% PI sites across 594 bp of CAD, several sites are present between sister species.

Table 1. Summary table of gene–genus statistics and neighbor-joining tree placement tests. PIS = parsimony informative site.

COI Barcode	Length	# PIS	% PIS	Conspecifics Properly Placed in NJ	Outgroups Properly Placed in NJ	Primers
Bembidion (Carabidae)	658	146	22.19	4/6	3/4	LCO1490, HCO2198 [51]
Monochamus (Cerambycidae)	659	120	18.21	46/62	3/4	LCO1490, HCO2198 [51]
Scolytus (Scolytinae)	612	248	40.52	31/39	4/4	1495b, rev750, F215, Rev453 [35]
Cyclorhipidion (Scolytinae: Xyleborini)	656	240	36.59	27/27	3/4	LCO1490, HCO2198 [51]; 1495b, rev750 [35]
Xyleborus (Scolytinae: Xyleborini)	649	175	26.96	22/22	3/4	LCO1490, HCO2198 [51]; 1495b, rev750 [35]
CAD Barcode						
Bembidion (Carabidae)	854	174	20.37	8/8	2/4	many [31,37]
Monochamus (Cerambycidae)	943	94	9.97	60/66	3/4	CD338, CD668, CD688 [33]

Table 1. *Cont.*

COI Barcode	Length	# PIS	% PIS	Conspecifics Properly Placed in NJ	Outgroups Properly Placed in NJ	Primers
Scolytus (Scolytinae)	471	157	33.33	48/50	4/4	CADforB2, CADfor4, CADrev1mod [52]
Cyclorhipidion (Scolytinae: Xyleborini)	594	70	11.78	11/11	2/4	CADforB2, CADfor4, CADrev1mod [52]
Xyleborus (Scolytinae: Xyleborini)	594	15	2.53	17/17	4/4	CADforB2, CADfor4, CADrev1mod [52]

Analysis of saturation within genera showed a 3rd codon position trend of COI saturating in transitions and transversions at a lower genetic distance than CAD (Figures 1 and S13). The genera that exceeded 15% pairwise divergence in COI showed transitions saturating. Transitions accumulate more quickly; thus, they begin to saturate before transversions. The CAD locus did not show evidence of saturation in any of the studied genera, and even with the inclusion of the outgroups, CAD remained unsaturated.

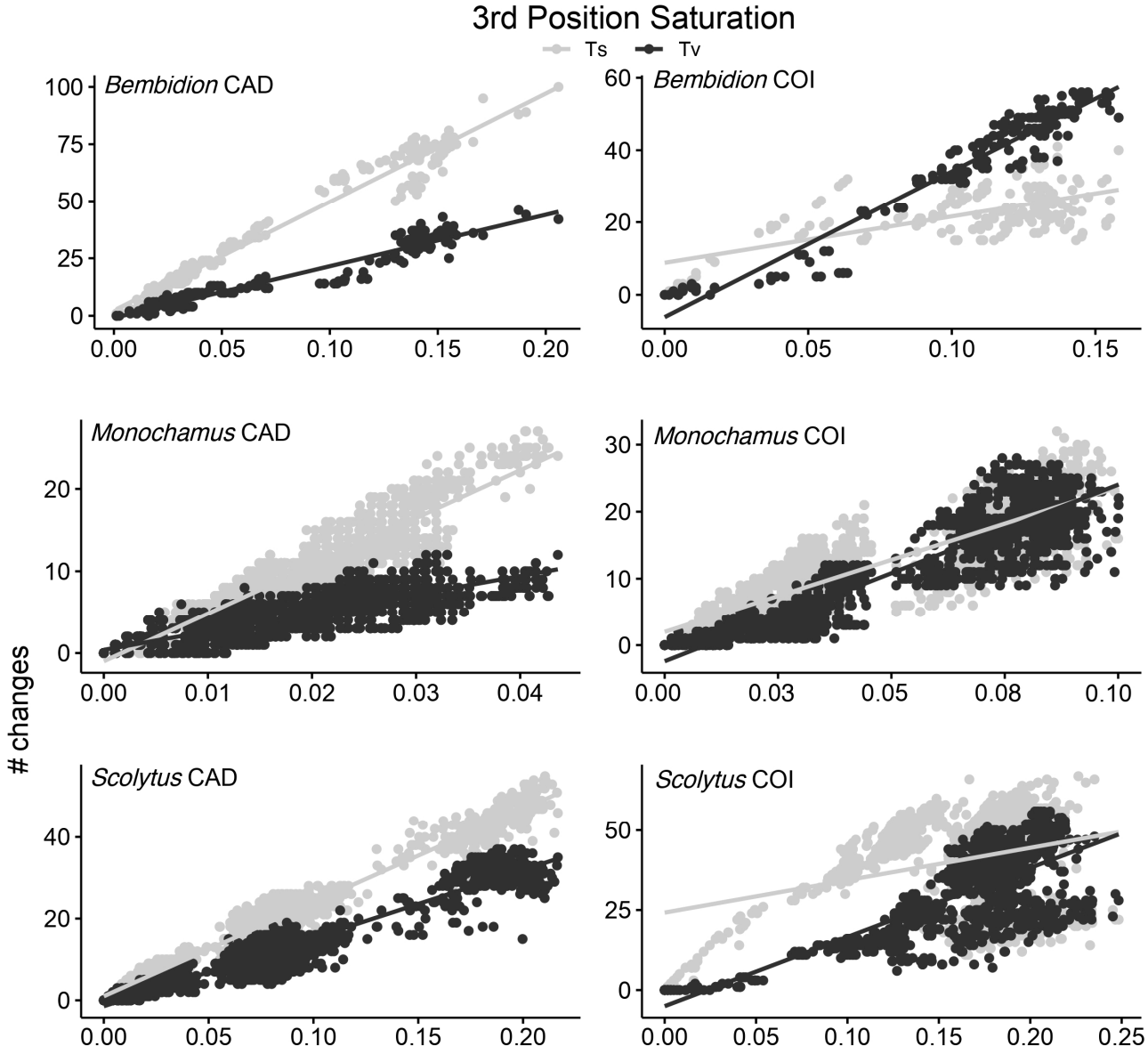

Figure 1. *Cont.*

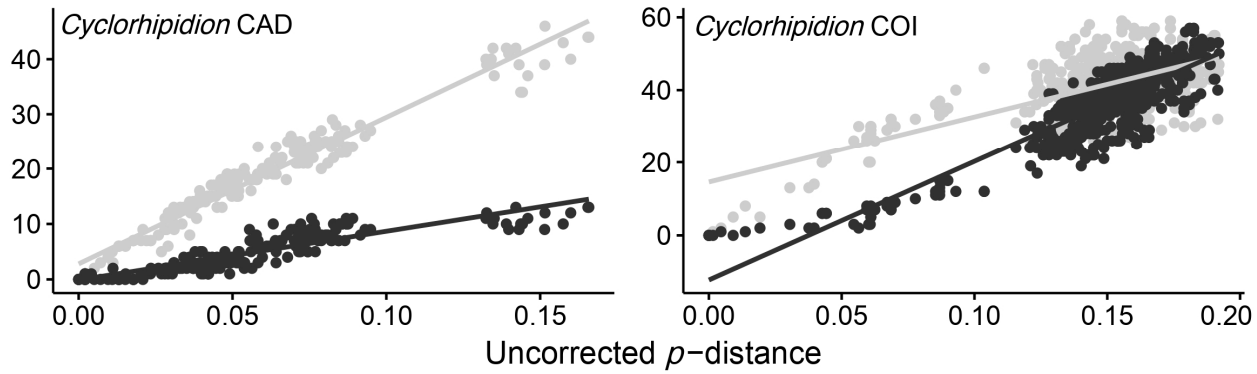

Figure 1. Barcode gene 3rd position saturation within each genus. The (**left**) column shows the plots for the CAD barcode and the (**right**) column shows the COI barcode. Lines of best fit are overlaid on the data points for transitions (grey) and transversions (black).

We tested for a traditional barcode gap between intraspecific and interspecific distances in both genes. In COI, we found a gap with no overlap only in *Xyleborus* (intraspecific quartile 3 = 0.09, interquartile 1 = 0.16; Figure 2); all other genera had at least an "ambiguous gap zone", (AGZ) where intraspecific and interspecific overlapped, but never beyond the interspecific Q1. For *Monochamus*, intra Q3 = 0.027–inter Q1 = 0.03, for *Scolytus*, intra Q3 = 0.02–inter Q1 = 0.16, for *Bembidion*, intra Q3 = 0.003–inter Q1 = 0.10, and for *Cyclorhipidion*, intra Q3 = 0.09–inter Q1 = 0.14. There were many examples of interspecific distances below 3% in the outbreeding genera. Remarkably, the inbreeding genera *Xyleborus* and *Cyclorhipidion* had a gap closer to 10%.

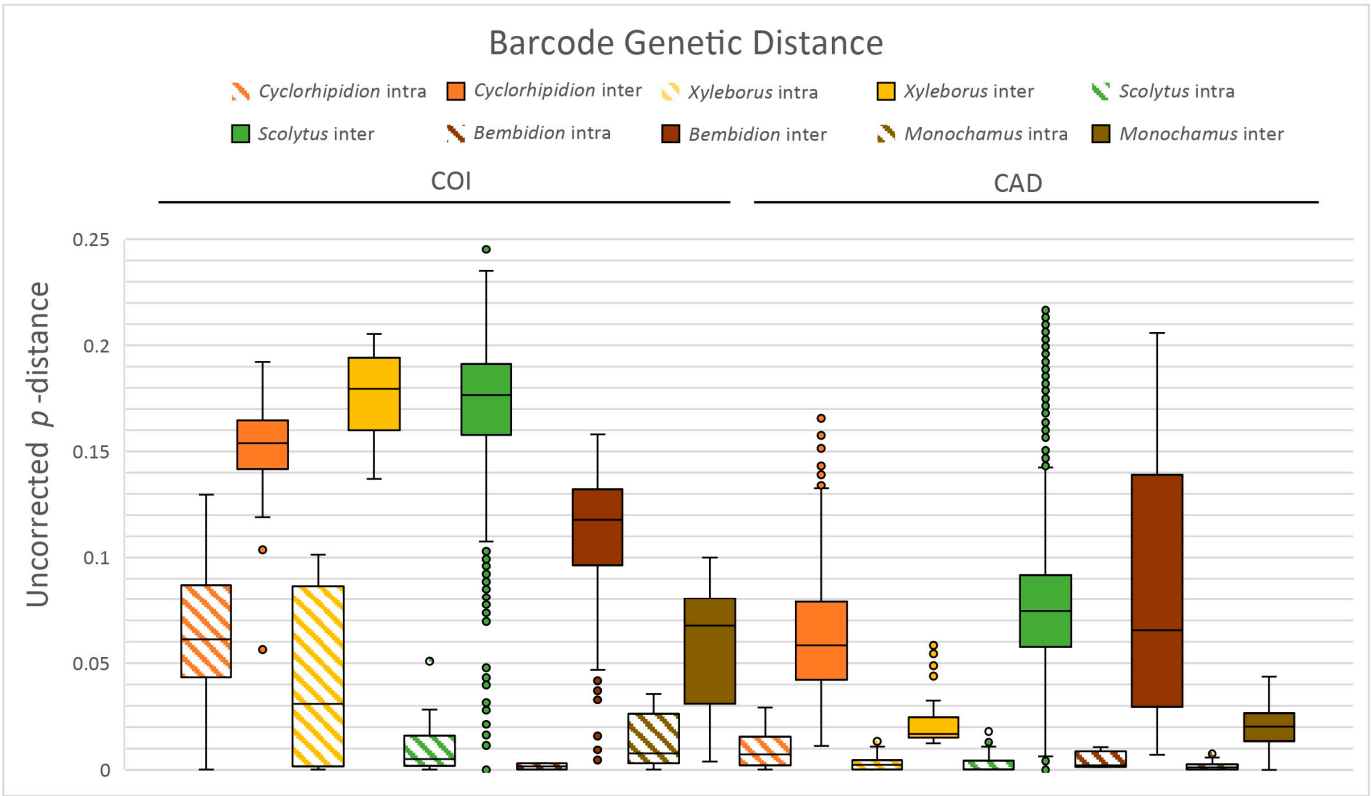

Figure 2. Pairwise distance box plot for COI (**left**) and CAD (**right**) data. intra = intraspecific comparison, inter = interspecific comparison.

Using the CAD marker, inbreeding and outbreeding genera were more similar than in COI. The presence of the AGZ was consistent with some minor overlap present at the extremes of the intra- and interspecific distance groupings (Figure 2). For *Monochamus*, intra Q3 = 0.006–inter Q1 = 0.014, for *Scolytus*, intra Q3 = 0.003–inter Q1 = 0.057, for *Bembidion*, intra Q3 = 0.009–inter Q1 = 0.029, for *Cyclorhipidion*, intra Q3 = 0.015–inter Q1 = 0.042, and for *Xyleborus*, intra Q3 = 0.003–inter Q1 = 0.015.

The ASAP automated method of delimiting taxa did not find the number of taxa defined by previous integrative analysis for any gene–genus combinations in this study (Table 2). This distance-based method depends on hierarchical clustering; thus, large interspecific distances as observed in the mitochondria of haplodiploid beetles should not complicate the analysis. The *Xyleborus* dataset had the most distinct barcode gap for each locus and the most intraspecific sampling. Yet, the ASAP best score overestimated the taxa present by six for COI and underestimated by five for CAD. Finding a CAD species "gap" was more difficult for the software, with a severe underscoring of taxon number in 60% of genera. In these, only three partitions were proposed which corresponded to the subfamily outgroup, tribe outgroup, and all the other taxa, including two genus-level outgroups. Unexpectedly, the ASAP estimate that matched the actual number of species in the datasets for several gene–genus combinations did not have a threshold value that fell within the AGZ (i.e., the intraspecific Q3 value to the interspecific Q1 value).

Table 2. Summary of ASAP species diagnosis method results with pertinent focus group statistics. Quartile values are only for comparisons within a genus.

	# Species in Tree (Including Outgroups)	Best ASAP Score # Species	Difference (# spp.)	Threshold Value for Best ASAP Score	ASAP Rank of Score Matching True Species #	Species Threshold of Correct ASAP Delim.	Empirical Intra Q3 Score	Empirical Inter Q1 Score	Is Best Species Threshold within Q3–Q1 Gap?
COI									
Monochamus	21	3	18	0.108388	7th	0.011198	0.027	0.031	no
Scolytus	38	35	3	0.04616	6th	0.018987	0.016	0.158	yes
Cyclorhipidion	21	31	10	0.040572	8th	0.109425	0.089	0.142	yes
Xyleborus	8	14	6	0.020031	2nd	0.112481	0.086	0.16	yes
Bembidion	20	16	4	0.027204	7th (19 spp.)	0.004669	0.003	0.096	yes
CAD									
Monochamus	21	3	18	0.126855	4th (19 spp.)	0.003886	0.002	0.014	yes
Scolytus	44	19	25	0.044184	6th (48 spp.)	0.003189	0.004	0.057	no
Cyclorhipidion	16	18	2	0.006112	3rd (17 spp.)	0.009061	0.015	0.042	no
Xyleborus	8	3	5	0.143786	4th	0.009514	0.004	0.015	yes
Bembidion	20	3	17	0.174192	tied-2nd	0.007185	0.009	0.029	no

3.2. Taxon Placement and Diagnostic Potential

Taxon placement at the species level was successful in the majority of cases for both genes (Figures S1–S10, Table 1), though issues related to mitochondrial DNA were recognized. Overlap was observed for each locus (Figure 2), with the AGZ present in both putative barcodes, and shifted closer to 1% in CAD. For both loci, there were interspecific distances measuring less than 1%. However, in COI, the majority were >14% in inbreeders and >3% in outbreeders. In CAD, Q1 interspecific values were above 1.3% and clear of the AGZ for each genus. The CAD neighbor-joining trees placed species in monophyletic groups better than those based on COI data (Table 1). With CAD, 91% or more were placed appropriately, while COI had three of five genera placing below 82% of species. Barcodes of shorter length (i.e., <50% of full-length for this study) could not be evaluated easily as many were the lone sample of a species, but the 274 bp COI *Scolytus rugulosus* (Figure S3) was placed far from its conspecific. When a test set of identified Eurasian *Monochamus* species was added to each gene tree for that genus, all nine individuals were placed with a conspecific.

The appropriate placement of outgroups in NJ trees was slightly more consistent in COI, where >75% were placed correctly (Table 1). All the genera incorrectly placed inside the target genus were part of the same tribe. In CAD analyses, *Cyclorhipidion* and *Bembidion* had both contribal outgroups placed within the focal genus. The *Monochamus* tree placed *Goes*, the same outgroup as in COI, within the genus. *Xyleborus* placed all outgroups

properly for CAD, while the outgroup genus *Euwallacea* was placed incorrectly in the COI tree. The "true" multigene published trees with substitutions modeled by partition always excluded the outgroup taxa except with *Monochamus*, where the *Goes* genus was not placed with high confidence either inside or outside *Monochamus* [33].

4. Discussion

Our analysis of the CPS region of the CAD gene among diverse Coleoptera shows that it is a suitable nuclear barcode for beetles. Nucleotide diversity at the species level, especially in third positions, allows for the discrimination of recently diverged taxa, even though no intron is usually present. CAD is a single-copy gene, and CPS barcode lengths under 1000 bp can be confidently amplified using a nested PCR procedure [31,33] and primers (Table 1). The locus is evolving quickly enough to discriminate species, while not so fast as to saturate within a tribe. This is in contrast to the saturated nucleotide COI differences among the clades of the diverse outbreeding genera *Bembidion* and *Scolytus* (Figure 1). This nuclear marker can also help delimit specific and generic boundaries of haplodiploid organisms (e.g., Scolytinae: Xyleborini), where COI divergence normally exceeds 10% and 15%, respectively [20]. The CAD barcode gap between intraspecific and interspecific distances has some overlap (Figure 2), similar to COI for the beetle groups explored. The gap zone for COI and CAD also varies between genera, indicating that a universal percent difference threshold would be inappropriate for either barcode. Still, CAD has species discrimination power as most conspecifics and test taxa were appropriately placed in neighbor-joining analyses (Table 1, Figures S1–S10). These factors make CAD a reasonable addition to any protocol for species diagnosis, species- to tribe-level phylogeny, or biodiversity monitoring.

Our comparative analyses indicate that a CAD barcode can remedy some problems observed with COI barcodes. Mitonuclear discordance is a concern revealed in cases where both mitochondrial and nuclear genes have been sampled [53,54] and is linked to the smaller effective population size of the mitochondrial genome. Introgression of mitochondrial DNA among species is a concerning issue and is likely common when congeneric species co-occur [55–58]. This introgressed mitochondrial DNA may artificially group the hybridizing species in a phylogeny. Mitochondrial introgression can also occur in nuclear genome-forming NUMT pseudogenes [59]. This phenomenon has been identified in several beetle groups [15,16] and is expected to be present across the order. This can lead to multiple clades representing one species in the COI gene tree; this has been shown in *Bembidion integrum*, which is known to have NUMTs. A nuclear gene population-level phylogeny, as we present with CAD, may potentially identify these instances. With these mitonuclear discordances recognized, they can be further investigated in the context of species morphology, ecology, and range overlap.

A CAD DNA barcode can also identify divergent patterns of COI variability. Deep divergence in COI, often near 10% within the haplodiploid Xyleborini species, would suggest an extraordinary number of cryptic species if applying a recommended 2–3% standard difference to delimit species [11]. However, in light of the CAD data, which delineate species at 2–3% over multiple haplodiploid genera, the high COI divergence was expected, given the biology of the beetles and the easily bottlenecked mitochondrial genome [20,53]. Thus, adding the CAD barcode can address the validity of tenuous COI diagnoses or cryptic species.

The saturation of nucleotide substitutions can cause problems with phylogenetic reconstruction and thus diagnostics. The primary mechanism is sites returning to their original base through multiple changes, which results in an underestimation of genetic distance and homoplasy [60]. As species divergence increases, so does this issue, and even intrageneric saturation in COI third positions is possible (Figure 1). Fortunately, we observed differentiation but not saturation in CAD, including at the subfamily outgroup level. The discrimination of higher-level taxa and the resolution of distant relationships have been successful with CAD in beetle phylogenetic studies [31,40]. This ability to

diagnose at a higher level can help identifiers when databases are sparse at the species level. However, users should be cautious as some outgroup genera were placed inside the ingroup in our barcode gene trees (Figures S1–S10). Individual saturation tests are feasible in barcode genes, but phylogenomic datasets will require automated tools, e.g., [61].

The process we have presented for species diagnosis using a CAD barcode is easily accessible for taxonomists already performing COI sequencing. The amplification of CAD has been successful across Coleoptera, and standard laboratory protocols have been established to produce small to multiple kb segments [30,62]. The biggest hurdle for diagnosticians is access to a representative CAD database of expertly identified species for comparison. Many sequences of CAD are available in GenBank at different levels of trustworthiness [63], and unlike in BOLD (available at https://boldsystems.org, accessed on 1 May 2023), voucher images are not included. Hence, verification of the identity of the specimen is difficult to impossible. However, a large, curated database like BOLD or adding CAD sequences to BOLD would alleviate this verification void and allow for simple phylogeny-based identification.

The stochastic nature of speciation may thwart the accuracy of single-gene barcoding analysis. Most specimens in our study were diagnosed correctly or were easily assigned to a mitochondrial issue, but the procedure was unreliable for several recently diverged taxa. Considering well-defined species, this issue could be caused by hybridization as discussed above, overlap between intra- and interspecific distances, or the retention of ancestral polymorphism (i.e., incomplete lineage sorting). Mitochondrial DNA, with a smaller effective population size relative to nDNA, should reflect species boundaries more quickly due to the extinction of shared mtDNA haplotypes [64]. However, rapid radiation can produce multiple species before even mtDNA can become monophyletic [55]. Shallow radiations of nuclear or mtDNA suffer from this problem, allowing shared mutations in two species, exclusive of one of their sister species [65]. Resulting differences in distance will cause mixing in barcode analysis, and methods that recognize synapomorphy will also be misled. Adding a CAD analysis could reveal this issue, but many nuclear genes are often needed to adequately address the problem [66]. In addition to rapid radiation problems, nuclear genes like CAD often maintain different alleles [26]; this heterozygosity can reduce informative sites and artificially increase nucleotide differences depending on how software treats ambiguous nucleotide coding. These factors further complicate automated methods; the ASAP software preferred overly conservative delimitations. Threshold methods like this seem to suffer when treating evolutionarily heterogeneous clades (e.g., the deeper splits of Palearctic *Monochamus* vs. the shallow splits of the Nearctic). When presented with an ambiguous identification, we suggest an iterative approach where the capacities of each barcode are used alongside other data like morphology to inform a diagnostic or delimitation result, e.g., [36,67].

We recognize several issues that can be addressed in future studies of the utility of barcodes. The first is the comprehensive sampling of intraspecific genetic diversity [13,39]. Our datasets had various amounts of intraspecific sampling, but likely none represented the maximum genetic distance among conspecifics due to research constraints (such as time and funding). These conspecifics likely have more genetic diversity than represented, which could result in an ambiguous gap for most species pairs. A second issue is polyphyly (as discussed in [55]), like that encountered in the North American clade of *Monochamus* (Figures S1 and S2). *Monochamus scutellatus* and its near relatives do not sort into monophyletic groups for either barcode, though morphology is distinct. When combined with low genetic divergence in that *Monochamus* clade, diagnostic methods cannot identify these species due to complicated speciation processes. While this issue is outside the scope of our investigation, creating a reference species tree using multiple loci could help in this situation [29]. Addressing the problem at the allele level could also help considering heterozygosity in some nuclear genes [33,68]. The ability of short sequences (i.e., "mini-barcodes") to function in the same way as full barcodes deserves further scrutiny. The potential for loss in discriminatory power is high and our shorter sequences, like *Scolytus*

rugulosus 72, did not place in accordance with published phylogenies. Finally, we are unsure how generalizable CAD barcoding will be to eukaryotes. While its utility has been shown in holometabolous insects, there may be issues (e.g., introns, primer sites) when targeting other insect groups and beyond. There may be potential for universal primer sites in CAD, but taxon-specific PCR primers are often necessary for taxa where these fail, e.g., [21,35]. This is a minor limitation for CAD, given that specific primers enhance the ability to amplify short DNA sequences from degraded tissues and target taxon-specific DNA from environmental samples. More data are needed to evaluate CAD's wide-scale potential as a comprehensive insect or eukaryote nuclear barcode.

5. Conclusions

Expanding gene sampling beyond one genetic locus and the mitochondrion can provide important corroborating evidence to delineate species and diagnose tissues lacking sufficient morphological characters (e.g., fragments, immatures, cryptic species). Additionally, adding nuclear evidence to standardized alpha diversity, eDNA metabarcoding, and invasive-species processing protocols will result in more robust conclusions of species composition. This is attainable in diverse and degraded specimens with current methodology that can take advantage of long-read sequencing (i.e., to address introns that may bias bulk sequencing output) and probe-based protocols. The CPS portion of the nuclear gene CAD is a locus capable of barcoding, identifying, and building phylogenies of varied ages in holometabolous insects, including the order Coleoptera [31,69,70]. Using CAD as an additional locus in species-level analyses or even as a replacement locus to diagnose deeper relationships can mitigate some of the problems that a strictly mitochondrial approach presents. By exploring varied datasets across beetles, we have shown that CAD can discriminate species synergistically with COI while being relatively easy to amplify and sequence using established Coleoptera primers. We propose integrating CAD into all Coleoptera barcoding and phylogenetic analyses to address the ubiquity of mitochondrial introgression, include an independent biallelic marker, and increase confidence in species-level delimitation and diagnosis.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d15070847/s1, Figures S1–S13: Figure S1: *Monochamus* COI tree; Figure S2: *Monochamus* CAD tree; Figure S3: *Scolytus* COI tree; Figure S4: *Scolytus* CAD tree; Figure S5: *Bembidion* COI tree; Figure S6: *Bembidion* CAD tree; Figure S7: *Xyleborus* COI tree; Figure S8: *Xyleborus* CAD tree; Figure S9: *Cyclorhipidion* COI tree; Figure S10: *Cyclorhipidion* CAD tree; Figure S11: *Monochamus* COI diagnostic test tree; Figure S12: *Monochamus* CAD diagnostic test tree; Figure S13: *Xyleborus* 3rd position saturation plots; Table S1: Taxon table.

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