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Estimating genetic diversity of abundant oceanic dolphins through repeated environmental DNA sampling

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Introduction: Estimating the genetic diversity of cetaceans at sea, particularly abundant social delphinids, can be difficult with traditional biopsy sampling of individuals. Environmental DNA (eDNA) metabarcoding has been shown to be a powerful tool for the identification of species assemblages and estimation of genetic diversity, especially in aquatic environments.

Methods: We collected 126 samples of seawater from within the immediate vicinity of schools of dolphins during 15 encounters with the four most common delphinid taxa in the waters around Santa Catalina Island, California, USA: long-beaked common dolphins (*Delphinus delphis bairdii*, $n = 8$), short-beaked common dolphins (*D. d. delphis*, $n = 3$), common bottlenose dolphins (*Tursiops truncatus*, $n = 2$), and Risso's dolphins (*Grampus griseus*, $n = 2$). Next-generation sequencing was used to assign Amplicon Sequence Variants (ASVs) of mitochondrial DNA to species using GenBank and a region-specific reference database.

Results: A total of 240 ASVs were resolved for the four species. ASV richness and the effective number of ASVs, or true diversity, measured as Hill numbers of order 1, were consistent with known characteristics of the four species. Despite collecting up to 12 samples from a single group, a rarefaction analysis indicated that the population diversity was not fully represented for the more abundant species (genus *Delphinus*), but were closely approximated for *G. griseus*.

Discussion: This study demonstrates the application of eDNA for estimating population genetic diversity of abundant species and makes recommendations for improving future studies to better capture this diversity in wild delphinid populations. This provides a more solid foundation for studies using eDNA to monitor these species, which often include those in close proximity to anthropogenic threats.

KEYWORDS

ASV, Catalina Island, genetic diversity, dolphin, eDNA, haplotype richness, metabarcoding, odontocete

Introduction

Genetic diversity is a central metric in conservation biology and the management of wild populations (Frankham, 1995; Lande, 1988). Quantifying the extent of diversity within a species can provide useful information about the size of the population, its demographic history, its potential vulnerability to future perturbations, and differentiation from neighboring populations (Nei et al., 1975; Petit et al., 1998; Hoffmann et al., 2017). To date, descriptions of genetic diversity and differentiation have been dependent on relatively small numbers of samples (Kalinowski, 2005; Luo et al., 2015; Phillips et al., 2019), which can be costly and take many years to accumulate. This has been especially true for species that are difficult to sample, such as cetaceans, which require dedicated vessel time, trained observers for locating animals, experienced vessel drivers to approach and work near animals, and trained personnel to collect biopsy samples or skin swabs (Noren and Mocklin, 2012).

If the objective of a study is to monitor changes in genetic diversity or evaluate low levels of genetic differentiation with neighboring populations of abundant species, with population sizes in the millions (e.g., common dolphins, *Delphinus* spp.), large sample sizes will be required to fully capture the genetic diversity (Phillips et al., 2019; Jefferson et al., 2024). Even abundant species with modest group sizes, such as bottlenose dolphins, (*Tursiops truncatus*) can be difficult to sample adequately without undue behavioral disturbance (Noren and Mocklin, 2012). Conversely, some social odontocetes, such as killer whales (*Orcinus orca*) and long-finned pilot whales (*Globicephala melas*) are strongly matrilineal and exhibit very low levels of mitochondrial DNA (mtDNA) diversity (Oremus et al., 2009; Morin et al., 2024) making it difficult to sample rare variants that might reflect social interchange.

Instead of relying on the direct collection of tissue samples, sampling the DNA shed into the environment (eDNA) has the potential to overcome some of these limitations (Goldberg et al., 2016; Deiner et al., 2017; Dickie et al., 2018; Adams et al., 2019; Zinger et al., 2019; Parsons et al., 2025). eDNA has been shown to be an effective tool for describing the relative composition of communities (Deiner et al., 2017), examining species distributions (Guillera-Arroita, 2017), and detecting rare or invasive species (Alter et al., 2022; Afonso et al., 2024). Additionally, unlike conventional biopsy sampling (Noren and Mocklin, 2012), the collection of eDNA from cetaceans is non-invasive and requires relatively little training.

While most eDNA studies have examined community diversity at the taxonomic level of species or higher, few have attempted to quantify or compare within-species genetic variability. If individuals within a population contribute relatively equally to the eDNA pool, or at least randomly with respect to their genotype, and if schools are not composed of highly related individuals, a given water sample should represent a random draw of the genetic diversity from the wider population. Although, in marine environments, eDNA can persist in the water well after the passage of source animals, sampling immediately after the passage of target species is expected to provide the greatest amount of obtained eDNA (Baker

et al., 2018; Robinson et al., 2023, 2025). Thus, the collection of repeated eDNA samples affords a more efficient, minimally invasive, cost-effective means to estimate population genetic diversity compared to the collection of biopsy samples (Baker et al., 2018, 2023).

Here, we present the results of such an experiment conducted around Santa Catalina Island, CA, USA. The study was designed to explore the utility of eDNA to quantify the genetic diversity of populations of the most frequently encountered dolphin species in the region. Recognizing that the use of eDNA in conservation biology is rapidly expanding and evolving, we also sought to develop procedures and explore analytical tools that could be used in future projects aimed at assessing or monitoring similarly abundant species.

Materials and methods

Field effort and sample collection

The study described here was a project secondary to a set of experiments to examine the behavioral response of cetaceans to naval sonar conducted around Santa Catalina Island, CA, USA during October and December of 2021. Details of this experiment are described in Southall et al. (2024). In this study, confirmed single-species schools of dolphins were sighted and then followed in a rigid-hull inflatable boat (RHIB) before, during, and after acoustic playbacks. During these focal follows, each hereafter referred to as an “encounter”, water samples were collected within 10 m of the school. Each encounter was focused on one of the four most common delphinids in the area: long-beaked common dolphins (*Delphinus delphis bairdii*, “Dbai”, $n = 8$), short-beaked common dolphins (*Delphinus delphis delphis*, “Ddel”, $n = 3$), common bottlenose dolphins (*Tursiops truncatus*, “Ttru”, $n = 2$), and Risso’s dolphins (*Grampus griseus*, “Ggri”, $n = 2$). The focal species was initially identified by personnel on the RHIB. For common dolphin schools, subspecies identification was confirmed with a review of morphological features in aerial images taken from unoccupied aerial system (UAS) flights being conducted at the same time (Durban et al., 2022; Leander et al., 2021). As far as could be ascertained from visual observations from nearby research vessels and UAS flight images, all schools sampled in this study were composed of a single species. Estimates of group size made from the RHIB were also evaluated from UAS analyses to yield a best group size estimate for each encounter.

Up to 12 water samples were collected during each encounter. Each sample was 2 L of seawater collected in two 1 L, wide-mouth, NalgeneTM bottles. Seawater was collected from the surface in the proximity of the dolphin groups being followed. Samples were taken at regular periods during encounters which lasted from one to 148 minutes from the first to the last sample (median = 57 minutes). After collection, each bottle was stored in a cooler with ice on the vessel. At the beginning of each day of sampling, a single 1 L bottle of tap water was placed in the cooler to serve as a negative field control. Additionally, at the beginning and end of the trip in

October and the beginning of the trip in December, approximately 1 L of tap water was filtered through the same apparatus for a total of 10 filter blanks.

Water samples were returned each evening to the Wrigley Marine Science Center on Santa Catalina Island, where the two 1 L bottles for each sample were filtered through a single 1-micron polycarbonate, track-etch filter (Whatman) using a low-pressure, oil-free vacuum pump (Air Cadet Single-Head Vacuum). The test filters and filter blanks were stored frozen (-20 degrees) in a 2 mL cryotube, with approximately 1 mL of Longmire's solution (Wegleitner et al., 2015) prior to eDNA extraction.

Here, we refer to the data derived from a single filter as a "sample". The only exception to this is the first two 1 L bottles collected on December 3, 2021, which were accidentally filtered separately. In the analysis below, the DNA extracted from these two filters were sequenced in the same run, and the ASVs and the resulting occurrence data from each were combined and treated the same as all other samples.

eDNA extraction and quantification

Approximately 500 μ L of the Longmire's buffer was removed from the tube and archived at -20 degrees. Total eDNA was extracted from the remaining buffer and filter by conventional phenol/chloroform/isoamyl methods (Renshaw et al., 2015; Baker et al., 2018). The resulting precipitated eDNA was resuspended in 100 μ L of TE buffer. Samples were extracted in batches of 12, based on the day of collection, and each extraction included one extraction blank. Following extraction, PCR inhibitors were removed or reduced using the OneStepTM PCR Inhibitor Removal Kit (Zymo Research) on all samples.

Metabarcoding library preparation and sequencing

An approximately 400 bp fragment of the mtDNA Control Region was amplified with high-fidelity polymerase (Kapa HiFi, Roche) in two rounds of nested PCR using methods described in Baker et al. (2023). In brief, an initial fragment, approximately 530 bp in length, was amplified using primers M13dlp1.5 and dlp5 and 20 PCR cycles. This product was then used as template in a second round of 20 PCR cycles to amplify a 390 bp fragment using primers dlp1.5 and dlp4. Amplification reactions were conducted in sets of 48, including 36 eDNA samples, extraction blanks, filter blanks, no-template controls, and a positive control. DNA from a tissue extraction of Hector's dolphin (*Cephalorhynchus hectori*) was used as a positive control because this species is endemic only to the coastal waters of New Zealand (Pichler et al., 2001). Nextera XT indices (Illumina) were added to clean individual amplicons and the resultant products were pooled into one library for paired-end 250 bp sequencing on an Illumina MiSeq using a Nano flowcell. Each batch of 48 samples and controls was amplified and prepared for sequencing twice. These duplicated library pools were then sequenced independently. Initial post-processing of each MiSeq run was done in the program Qiime 2 (Bolyen et al., 2019), where raw reads were demultiplexed, then trimmed to remove primer

sequence and denoised. The paired-end reads were merged using the *dada2* plugin (Callahan et al., 2016).

Negative control correction

Read counts for ASVs identified in water samples were then corrected to account for their occurrence in the set of negative controls used throughout the process. For any ASV found in a negative control, we subtracted its read counts from the same ASV found in a water sample collected as part of the sampling or lab process that it was related to. For example, if an ASV was found in a negative control from a particular extraction batch, we would subtract the read counts of that ASV from the same ASV found in water samples that were extracted in the same batch. This read count subtraction occurred where necessary for all negative controls. If the same ASV was found in both runs of a particular negative control, then the mean of the read counts between the two runs was subtracted. If, after this serial subtraction, the read counts for an ASV in a particular water sample was zero or less, it was considered a full contaminant and removed from that sample.

ASV species assignment

We developed a multi-step pipeline for resolving ASVs produced by QIIME/DADA2 and assigning them to species with high confidence (Supplementary Figure SM1; Supplementary Table SM1). Step 1 was to run two BLAST searches of all ASV sequences; one on NCBI GenBank, and another on a local reference database of mtDNA control region sequences from delphinids known to occur in the Southern California Bight (SCB), around Santa Catalina Island (number of reference haplotype sequences shown in parentheses): *Delphinus delphis bairdii* (42), *Delphinus delphis delphis* (151), *Grampus griseus* (69), *Lissodelphis borealis* (27), *Aethalodelphis obliquidens* (138), *Orcinus orca* (86), *Steno bredanensis* (52), *Stenella coeruleoalba* (111), *Tursiops truncatus* (40). For this study, these SCB reference sequences were submitted to GenBank and accession numbers for all sequences are provided in Supplementary Table SM2. Both GenBank and local BLAST searches were run with *blastn* in the BLAST+ v.2.16.0 suite (Camacho et al., 2009). Only the top 10 hits that matched a cetacean reference sequence at $\geq 85\%$ of its length, and had e-values $\leq 10^{-100}$, were retained.

For Step 2, we only retained ASVs from Step 1 that had BLAST hits from either database that were delphinids occurring in the SCB, matched a reference sequence ≥ 345 base pairs and $\geq 99\%$ of the reference sequence length, and matched at $\geq 95\%$ of the ASV sequence. If a hit matched a reference sequence at only $\geq 95\%$ of the reference sequence length, but satisfied all of the other criteria, then the hit had to be in the top 5 and have a bitscore ≥ 550 . Only ASVs and the hits meeting these criteria were retained.

In Step 3, for ASVs that were retained from Step 2 and had 100% identity matches in the retained BLAST hits, we used the species from those hits as species assignments for those ASVs. For the remaining retained ASVs, we created a Random Forest (RF) classification model using the SCB reference sequence dataset and the *randomForest* v4.7-1.1 package (Liaw and Weiner, 2002) in R

v.4.4.0 (R Core Team, 2024). For the RF model, we aligned all SCB reference sequences with all ASVs that were retained after Step 2. We then trimmed the alignment so all sequences were of the same length. The RF model was then built from the trimmed SCB reference sequences, only using sites that were variable in at least two sequences. Sample sizes for each species in each tree in the RF model were balanced to use 50% of the smallest sample size, as described in Archer et al. (2017). A total of 10,000 trees were grown with the number of sites evaluated at each node left at the *randomForest* default.

In Step 4, we assigned ASVs with confidence using the RF model in Step 3. For this step, we used the distributions of out-of-bag (OOB) assignment probabilities for each SCB reference species. For each of these distributions, we set a threshold as the smallest assignment probability of the top 90th-percentile of the reference sequences. ASVs that were assigned to a species in the RF model with probabilities equal to or greater than this threshold were considered to be similar enough to a majority of the reference sequences to accept that assignment.

The fifth and final step was to remove unique ASVs that may have been due to errors in the sequencing process. We removed any ASV with a species assignment from the RF model that was a unique haplotype only because it differed from the nearest reference haplotype at sites that were otherwise fixed in the rest of the reference sequences. The assumption is that a previously unsampled haplotype is most likely to result from substitutions at known variable sites rather than one or more substitutions at otherwise invariant sites.

Genetic diversity

We calculated two measures of genetic diversity based on the validated ASVs. The first, richness (R), is simply the absolute number of ASVs observed in a sample (Nei et al., 1975; Petit et al., 1998). The second, the effective number of ASVs (D), is a true measure of diversity that takes into account the relative frequency of ASVs (Jost, 2007; Chao et al., 2010; Jost et al., 2010). For this study we used read counts to represent the frequency of occurrence of each ASV within a sample. However, to normalize read counts across samples and MiSeq runs, we computed the proportion of each ASV within an encounter as the sum of read counts of that ASV divided by the sum of read counts of all ASVs assigned to SCB cetacean species within that encounter. For each encounter, we then computed D as the Hill number of order 1 given the distribution of the proportion of reads composed of each ASV (Jost, 2007). In cases where we computed D for multiple encounters, we summed these proportions across encounters. Both R and D were calculated with the package *sprex* v.1.4.2 (Archer, 2024) in R v.4.4.0 (R Core Team, 2024).

In order to examine the effect of the number of samples collected within an encounter on estimates of genetic diversity for those encounters, we randomly sampled all unique combinations of 1 to k_i samples within each encounter, where k_i is the number of samples in the i -th encounter. For each random subset of samples within the

encounter, we computed R and D as above for ASVs from each species. We also conducted the same procedure for all unique combinations of 1 to N encounters. For this latter resampling procedure, we examined how true genetic diversity (D) changes as more ASVs were encountered by summarizing the distribution of D for each unique value of R within each eDNA species.

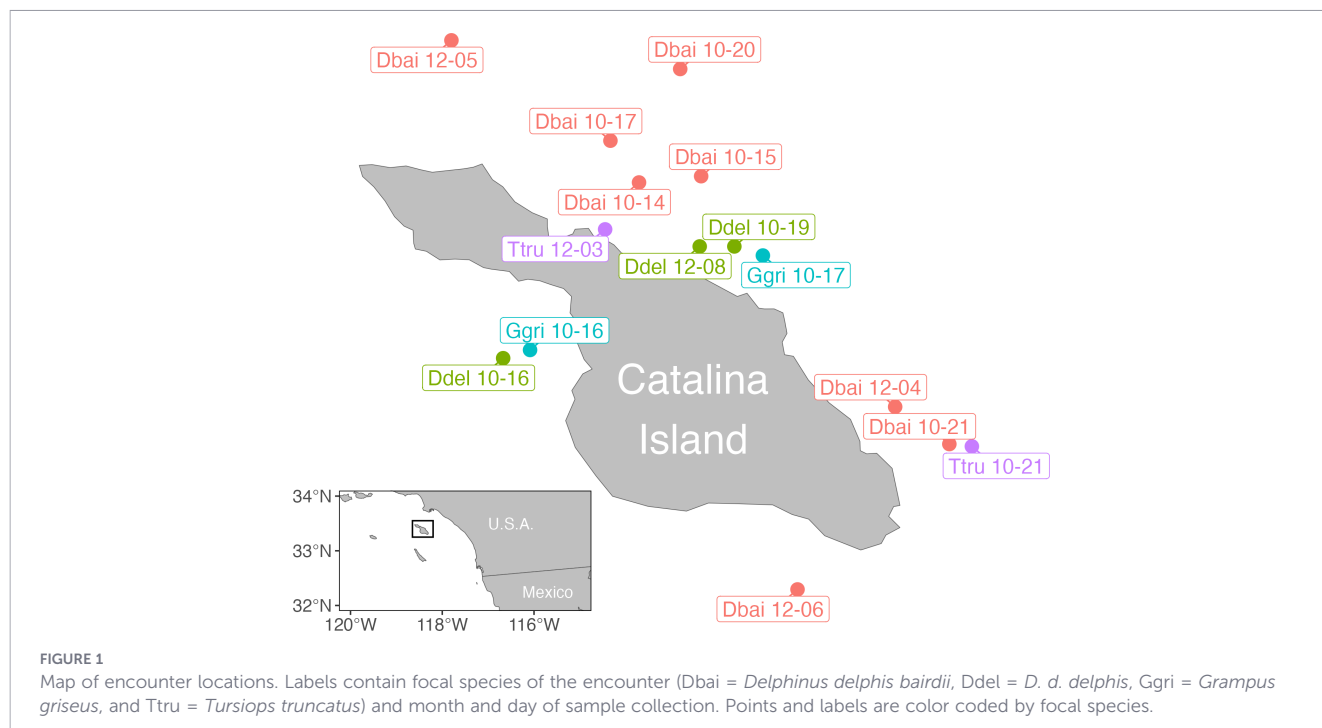
Finally, we examined the effect of the number of encounters, and samples within each encounter, on estimates of overall genetic diversity for each eDNA species. For this procedure, we first randomly selected all unique combinations of 1 to N encounters, where N is the total number of encounters of a given focal species. Within each encounter in a given random subset, we then randomly selected 1 to the smallest number of samples for all encounters in the subset. We did this for all unique combinations of a focal species' encounters and samples or 1000 random draws if there were more than 1000 unique combinations. As above, we computed R and D for ASVs from each eDNA species in each of these randomly selected subsets of encounters and samples.

For both resampling procedures, we compared the overall value of richness and effective number of ASVs with the distributions of these values produced in the within-encounter resampling and the encounter $\times$ sample resampling. We report the proportion of these distributions greater than or equal to the values obtained using all samples together.

Results

A total of 126 water samples were collected over 12 days of effort and 15 encounters occurring in October and December, 2021 during daily surveys within 10 km of Santa Catalina Island, CA, USA (Figure 1, Table 1). After filtering, extraction, and library prep, a total of 8 MiSeq Nano runs were conducted, with an average of 36 samples per run. On average, approximately 30 samples in a run were from extracted filtered seawater, five from negative controls from various stages in the process (collection, extraction, and sequencing), and one positive control. Each run generated approximately 870K (minimum = 537,270, maximum = 1,125,614) reads and an average of 147 unique ASVs. On average, approximately 96% of the reads in each run ($n \cong 831K$) were of ASVs from the filtered seawater samples, with the remaining 4% of reads being associated with negative and positive control samples. There were approximately 127 unique ASVs across the filters sequenced in each run.

The level of contamination as measured by the presence of ASVs in the various negative controls was low. On average, about 5% ($n \cong 7$) of the ASVs in a sequencing run were found in one or more negative controls. However, only approximately 1.2% of the reads ($n \cong 9200$) in a sequencing run were from ASVs found in negative controls. Across all eight sequencing runs, a total of 31 ASVs found in negative controls were also found in a seawater filter sample in the same sequencing run. This was approximately 62% of all ASVs found in negative controls.



Using Hector's dolphin DNA (GenBank Accession # AF057995) as a positive control produced approximately 18 ASVs per sequencing run. Approximately one third (34%) of the ASVs found in each positive control sample had a high probability (> 95%) match to Hector's dolphin sequences in a BLAST search, but only 18% (3–4 ASVs per

run) were exact matches to a Hector's dolphin Genbank sequence. However, these ASVs that matched the Hector's dolphin GenBank sequence were represented by an average of 86% of the reads across the positive control samples. An average of 14% of the positive control reads were ASVs that had high probability BLAST matches to other Hector's dolphin sequences. The remaining ASVs that did not have high probability Hector's dolphin matches composed an average of 4% of the reads across the positive control samples and were thus regarded as contaminants or polymerase error.

Detection of ASVs in replicate sequencing runs of extracts from the same filter was relatively low. On average, only 17% of all ASVs found in a filter were found in both of the duplicate sequencing runs. However, 26% of ASVs assigned to a SCB cetacean species in a filter were found in both runs. Although the proportion of reads accounted for by each ASV in the replicate runs varied, the average difference in the read proportion for ASVs found in both runs was 0.03% and not significantly different from zero.

Species assignment

A total of 836 ASVs were identified from initial analyses. Of those, 636 ASVs were identified by BLAST as cetaceans, either in GenBank or in our local database of SCB cetaceans. 503 ASVs were then identified as likely to be SCB odontocetes. We then assigned 240 ASVs to species that either matched perfectly to a reference sequence or were determined to be valid ASVs based on the location of nucleotide substitutions. All 240 of these ASVs were assigned to one of the four focal species (Table 2, Supplementary Figure SM1). Here on, we refer to species identification of the ASVs as the "eDNA species" to differentiate them from the "focal species" of each encounter.

TABLE 1 Summary of focal species encounters.

Focal species	Date	Best	Minutes	Samples
Dbai	10-14	52	106	12
Dbai	10-15	100	57	12
Dbai	10-17	80	89	3
Dbai	10-20	200	110	12
Dbai	10-21	260	148	10
Dbai	12-04	250	65	12
Dbai	12-05	10	23	4
Dbai	12-06	150	27	12
Ddel	10-16	200	70	11
Ddel	10-19	40	128	12
Ddel	12-08	150	27	12
Ggri	10-16	20	1	1
Ggri	10-17	20	55	9
Ttru	10-21	20	3	2
Ttru	12-03	25	11	2

Shown are the focal species of the encounter (Focal: Dbai, *Delphinus delphis bairdii*; Ddel, *Delphinus delphis delphis*; Ggri, *Grampus griseus*; Ttru, *Tursiops truncatus*), the collection date in 2021 as MM-DD (Date), the best estimate of the school size (Best), the minutes elapsed between the first and last sample (Minutes), and the total number of samples collected (Samples).

TABLE 2 Summary of ASV species assignments.

eDNA species	Encounters	ASVs	100% BLAST	New	Samples	Mult. samples	Median reads	All eDNA	All reference	Trimmed eDNA	Trimmed reference
Dbai	14	122	29	69	92	41	838	122 18.9	62 33.6	117 18.9	38 20.7
Ddel	11	87	22	22	59	22	316	87 16.8	165 136	81 16.7	144 109.8
Ggri	12	16	1	10	25	10	1,252	16 4.8	15 13.3	16 4.8	14 13.7
Ttru	5	15	5	5	11	3	401	15 3.6	36 28.7	14 3.6	33 24.4
All	15	240	57	106	120	76	664				

Shown are the species the ASVs were assigned to (eDNA Species: Dbai, *Delphinus delphis bairdii*; Ddel, *Delphinus delphis delphis*; Ggri, *Grampus griseus*; Ttru, *Tursiops truncatus*), the number of encounters containing ASVs of this species (Encounters), the number of ASVs assigned to each species (ASVs), the number of ASVs with 100% BLAST matches (100% BLAST), the number of new ASVs that were not in GenBank or our local reference datasets (New), the number of samples with ASVs of this species (Samples), the number of ASVs of this species in multiple samples (Mult. Samples), and the median read count across ASVs of this species (Median Reads). The last four columns are diversity metrics (Top: Richness, Bottom: Effective number of ASVs) for all eDNA sequences (All eDNA), all reference sequences (All Reference), eDNA sequences trimmed to the same length (Trimmed eDNA), and reference sequences trimmed to the same length (Trimmed Reference).

eDNA species diversity

Overall, long-beaked common dolphins (*Delphinus delphis bairdii*) exhibited the largest measures of ASV richness and diversity, followed by short-beaked common dolphins (*Delphinus delphis delphis*) (Figure 2, Table 3). On the other hand, Risso's dolphins (*Grampus griseus*) and bottlenose dolphins (*Tursiops truncatus*) had considerably lower and similar measures of richness and diversity. Overall richness (R) was greater in the reference sequences than in the ASVs for short beaked common dolphins and bottlenose dolphins. However, ASV richness was almost 2x greater than reference richness for long-beaked common dolphins, but only slightly greater for Risso's dolphins. For all four species, true diversity (D) was greater in the reference sequences than in the ASVs (Figure 2, Table 3). However, it was very similar for long-beaked common dolphins (trimmed eDNA = 19, trimmed reference = 21), while most dissimilar for short-beaked common dolphins (trimmed eDNA = 17, trimmed reference = 110).

In each of the 15 encounters, the ASVs corresponding to the focal species had the greatest diversity (Figure 3, Table 3). However, there was considerable variability in diversity across encounters for each focal species and some ASVs assigned to non-focal eDNA species. The long-beaked common dolphin encounter on October 20 had the greatest diversity, recovering 62 ASVs composed of 55 long-beaked common dolphin ASVs, 6 short-beaked common dolphin ASVs, and 1 Risso's dolphin ASV. Long-beaked common dolphin ASVs were present in all encounters but one, a bottlenose dolphin encounter on December 3. It is notable that Risso's dolphin ASVs were regularly found in focal encounters of the two common dolphin species, but were absent in the two focal encounters with bottlenose dolphins. Conversely, bottlenose dolphin ASVs were relatively rare in non-bottlenose dolphin focal encounters.

When diversity is expressed as a percent of the overall diversity within each encounter, values of R and D are similar to each other for eDNA species within encounters (Figure 4). However, there is a tendency for % R to be greater than % D when the eDNA species is the same as the focal species, with the reverse being true when the eDNA species is not the focal species. Additionally, there is a suggestion of an overall decrease in % R and % D for the eight long-beaked common dolphin encounters through time (from October 14 to December 6).

There was also considerable variability in the relative frequency of ASVs of each eDNA species from sample to sample, within encounters (Figure 5). For example, although most samples in long-beaked common dolphin focal encounters contained long-beaked common dolphin eDNA, two samples in the December 5 encounter, and three samples in the December 6 encounter did not. Similarly, although Risso's dolphin ASVs were present in many encounters, albeit sporadically and at low levels, they were not present in three of the nine samples in the October 17 encounter where Risso's was the focal species.

Resampling

By examining combinations of random samples within encounters, we see that estimates of eDNA diversity increase as

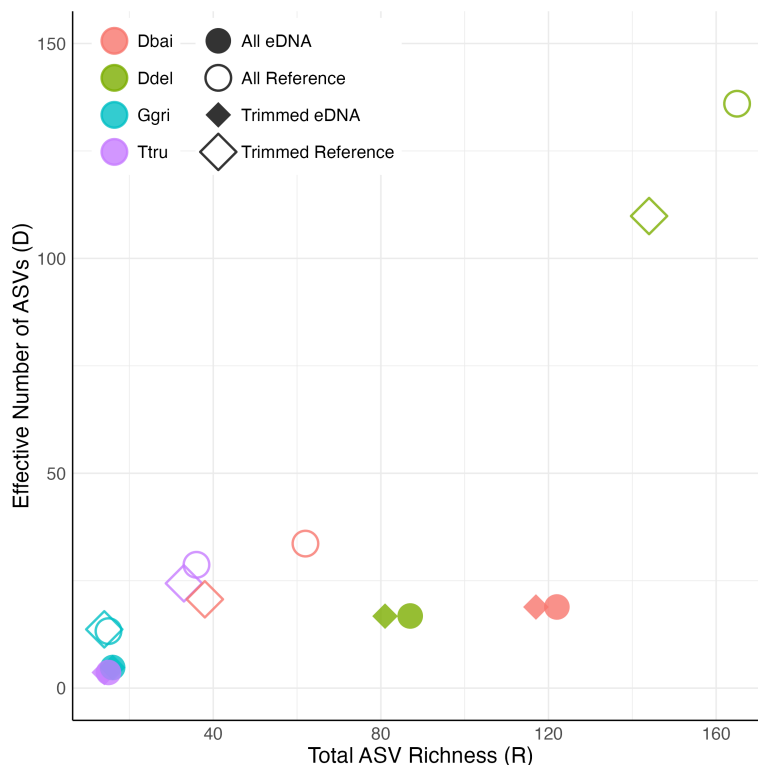


FIGURE 2

Total ASV richness (x-axis) and effective number of ASVs (y-axis) across all eDNA samples (closed symbols) and within the reference dataset (open symbols) for the four focal species (Dbai = *Delphinus delphis bairdii*, Ddel = *D. d. delphis*, Ggri = *Grampus griseus*, and Ttru = *Tursiops truncatus*). Both metrics are computed for the full-length sequences for both eDNA and reference datasets (circles) as well as sequences trimmed to the same length (diamonds) within each species.

more samples are taken in an encounter (Supplementary Figure SM2). In focal encounters of the two common dolphin species, the diversity (D) of the same eDNA species does not reach the overall diversity even with the maximum number of samples collected. However, diversity of Risso's dolphin eDNA within Risso's dolphin focal encounters does seem to encompass the overall diversity after about six samples. There is some indication that eDNA diversity in long-beaked common dolphin encounters begins to reach an asymptote at 12 samples, while diversity seems to be linear with respect to the number of samples for short-beaked common dolphins. We also note that Risso's dolphin eDNA diversity in long-beaked common dolphin focal encounters remained low and relatively consistent even with increasing numbers of samples. Conversely, there seems to be a low level of long-beaked and short-beaked common dolphin diversity detected in Risso's dolphin focal encounters that increases as more samples are collected.

Likewise, in combinations of randomly drawn encounters, the effective number of ASVs increases with increasing ASV richness for each eDNA species (Figure 6). However, as with the within-encounter resampling, there is evidence of an asymptote of diversity for long-beaked common dolphins with increasing richness that is not present for short-beaked common dolphins. The asymptote is also evident for Risso's dolphins. The lack of any clear pattern for bottlenose dolphins is likely a product of the relatively small number of eDNA detections of this species across all encounters.

Permutations of both encounters and samples suggest that for all species, it is not possible to recover the overall ASV richness (R)

by combining data from fewer encounters or fewer samples within those encounters (Supplementary Figure SM3). Only a few simulations recovered more than half of the overall richness, especially for ASVs of eDNA species sampled within focal encounters of the same species. Conversely, the overall effective number of ASVs (D) could be approximated more closely with fewer encounters and samples per encounter (Supplementary Figure SM4). The clearest example is seen in the long-beaked common dolphin ASVs from the long-beaked common dolphin focal encounters (upper left, Supplementary Figure SM4). Here, out of eight long-beaked common dolphin encounters, the overall diversity of long-beaked common dolphin ASVs could be recovered with approximately five encounters and 11 samples per encounter, or four encounters and 12 samples per encounter. Conversely, subsampling short-beaked common dolphin encounters could, at best represent approximately 25% of the overall effective number of short-beaked common dolphin ASVs.

Discussion

Sequences derived from eDNA are rapidly becoming effective tools for the detection, identification, and monitoring of multiple species of cetaceans around the world (Foote et al., 2012; Baker et al., 2018; Robinson et al., 2023; Zhang et al., 2023; Afonso et al., 2024; Parsons et al., 2025; Bennington et al., 2024). While much of

TABLE 3 Summary of genetic diversity in focal species encounters.

Focal species	Date	Diversity					
		Mean	Total	Dbai	Ddel	Ggri	Ttru
Dbai	10-14	31.1 8.6	49 9.5	46 9.4	2 1.1	1 1	
Dbai	10-15	31.1 8.6	25 9.3	22 8.4	2 1.9	1 1	
Dbai	10-17	31.1 8.6	23 10.2	17 8.4		6 3.1	
Dbai	10-20	31.1 8.6	62 17	55 16.8	6 2.5	1 1	
Dbai	10-21	31.1 8.6	25 6.6	16 6.1	5 2.7	1 1	3 2.3
Dbai	12-04	31.1 8.6	30 7.2	21 7.2	5 3.2	1 1	3 3
Dbai	12-05	31.1 8.6	4 1.4	3 1		1 1	
Dbai	12-06	31.1 8.6	31 7.5	6 3.2	17 5.2	6 2	2 2
Ddel	10-16	32 6.9	15 3.3	4 2.5	11 3.2		
Ddel	10-19	32 6.9	36 4.9	2 1.7	32 4.9	2 1.8	
Ddel	12-08	32 6.9	45 12.6	8 3.6	34 9.9	3 1.6	
Ggri	10-16	9.5 4.1	2 1	1 1		1 1	
Ggri	10-17	9.5 4.1	17 7.1	5 2.8	5 2.8	7 2.8	
Ttru	10-21	8 2.3	11 3.5	2 1.2	1 1		8 3
Ttru	12-03	8 2.3	5 1.1				5 1.1

Shown are the focal species (Focal Species: Dbai, *Delphinus delphis bairdii*; Ddel, *Delphinus delphis delphis*; Ggri, *Grampus griseus*; and Ttru, *Tursiops truncatus*), the collection date in 2021 as MM-DD (Date), the mean diversity across samples in the encounter (Mean), total diversity for the encounter (Total), then diversity of each of the four eDNA species in the encounter. For measures of diversity, top value is ASV richness (R), and bottom value is effective number of ASVs (D).

the focus of recent work with eDNA has been on detection of rare species, in this study, we have explored the utility of eDNA to quantify and compare genetic variability among abundant, social delphinids. Historically, these data were only available after the costly accumulation of individual tissue samples collected over long periods of time (Noren and Mocklin, 2012). A general rule of thumb has been that a minimum of 30–50 samples per strata are necessary to capture genetic diversity within wild populations (Kalinowski, 2005; Willing et al., 2012; Hale et al., 2012). However, given that genetic diversity is a function of effective population size (Kimura, 1983; Frankham, 1995), this general rule of thumb may be insufficient to represent the genetic diversity of social, oceanic delphinids, such as species in the genera *Stenella* or *Delphinus*. These species can have census population sizes in the hundreds of thousands to the millions (Barlow and Forney, 2007; Wade et al., 2007; Carretta et al., 2024). The results of our study suggest that population genetic diversity of these abundant species can be more efficiently and cost-effectively quantified via repeated eDNA sampling.

The two measures of genetic diversity that we estimated, richness and true diversity (the effective number of ASVs as described by the Hill number of order 1; Hill, 1973) were selected for the complementary information they provide about these populations. Allelic richness, or the number of alleles maintained at a locus, has been recognized as the most frequently reported and simplest measure of genetic diversity (Allendorf, 1986; Kalinowski, 2004). Allelic richness has been shown to be more sensitive to population bottlenecks than differences in heterozygosity (Cornuet and Luikart, 1996; Petit et al., 1998) and thus can also be used to infer patterns of population establishment (Castric and Bernatchez, 2003). Additionally, because the number of alleles at a locus can be used as a direct measure of the evolutionary potential of a population, it is also considered an important metric from a conservation and management standpoint (Leberg, 2002; Petit et al., 1998).

Haplotypic diversity (i.e., haploid heterozygosity) is a commonly used measure of diversity for mtDNA sequences (Nei and Tajima,

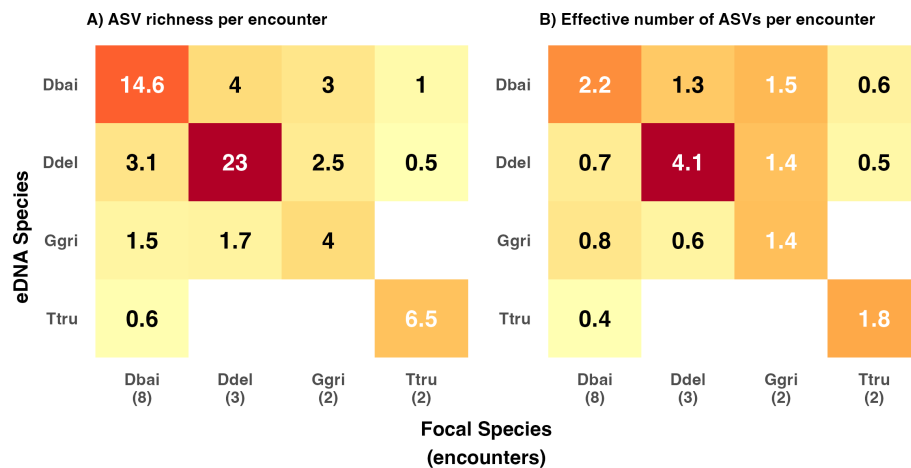


FIGURE 3

ASV richness (A) and effective number of ASVs (B) per encounter by focal species (x-axis) and eDNA species (y-axis). Species labels are Dbai = *Delphinus delphis bairdii*, Ddel = *D. d. delphis*, Ggri = *Grampus griseus*, and Ttru = *Tursiops truncatus*. The number of encounters for each focal species are given in parentheses on x-axis.

1981; Nei, 1987). However, it has been demonstrated that this measure does not behave as a proper diversity metric as it does not scale linearly and is not comparable between taxa or studies (Jost, 2006, 2007; Chao et al., 2014; Jost et al., 2018). Conversely, a large body of literature has shown that gene diversity as measured by Hill numbers has all the desirable properties of a diversity estimator, quantifying the effective number of elements represented in a frequency distribution (Hill, 1973; Jost, 2006, 2007; Chao et al., 2014). Given the different population sizes and demographic histories of the four focal species in our study, this metric allowed us to appropriately compare estimates within species across encounters as well as among species.

In abundant species, we expect the true frequency distribution of ASVs within a species to have a very long tail, with many ASVs present at low frequencies (Nei, 1987; Nei and Kumar, 2000). This means that repeated water sampling would have to be extensive to uncover the complete ASV richness in the population from eDNA. Given this, the observed number of ASVs (richness) can be considered to be more of a measure of sampling effort, especially early in the sampling process. However, as serial sampling continues, the rare ASVs, which add more to richness, do not contribute much to estimates of true diversity. Thus, we expect corresponding estimates of true diversity to asymptote towards the real population diversity as the true ASV frequency distribution is uncovered. It is this signal that is described by our discovery curve in Figure 6.

From this curve, we observed that our sampling captured nearly all the genetic diversity of long-beaked common dolphins, but not of short-beaked common dolphins. Even though total diversity was assessed across all 15 focal encounters, eight of these encounters were with long-beaked common dolphins, while only three were with short-beaked common dolphins. In the California Current, long-beaked common dolphins occur within 95 kilometers of the coast and range from central California to Baja California with an estimated population size of approximately 83,000 (Barlow and Forney, 2007; Carretta et al., 2024). Conversely, short-beaked common dolphins have a much larger range, occurring further offshore out to at least 560 kilometers from the coast, with sightings as far north in the California Current as Washington and south to mainland Mexico.

Estimated population sizes are over 1 million (Barlow and Forney, 2007; Carretta et al., 2024). Thus, while both species occur around Catalina Island, our study area is more within the core of the range of the less abundant long-beaked common dolphins, which would explain how we could capture more of the genetic diversity of this species, compared to short-beaked common dolphins.

Although we only had two focal encounters with Risso's dolphin (*Grampus griseus*), we found strong evidence that we were capturing the genetic diversity of this species within the study area (Figure 6). While they range along the US west coast, from northern Washington to southern California, they are most abundant from central to southern California and are particularly frequent within the Channel Islands in the Southern California Bight (Barlow, 2016; Carretta et al., 2024). Although their range overlaps largely with that of short-beaked common dolphins, the population size of Risso's dolphins within the California Current is considerably less, estimated at approximately 6,000 individuals (Barlow, 2016; Carretta et al., 2024).

At least one Risso's dolphin ASV was present in most encounters, regardless of the focal species, and ASVs from Risso's had the highest median read count across all encounters. This could simply be a result of the relatively high usage of *Grampus* in the area immediately around Catalina Island. However, *Grampus* may also be releasing relatively more DNA in the water than other species due to the physicality of their social interactions and raking behaviors (MacLeod, 1998; Hartman et al., 2016; Mariani et al., 2016). Given that cetaceans naturally slough their skin on a regular basis, making them suitable candidates for detection by eDNA (Amos et al., 1992; Baker et al., 2018), the continuous tooth raking behavior of *Grampus* may lead to the species releasing relatively more DNA into the water than other species, especially if they are frequently using a local area.

In general, we should not expect the DNA shedding and sampling availability rates of the four focal species to be the same. Although there is no direct comparison of these rates in cetaceans, the evidence from other species in the aquatic environment suggests that shedding is likely to be affected by a combination of differences in physiology, school size, and behavior (Klymus et al., 2015; Barnes

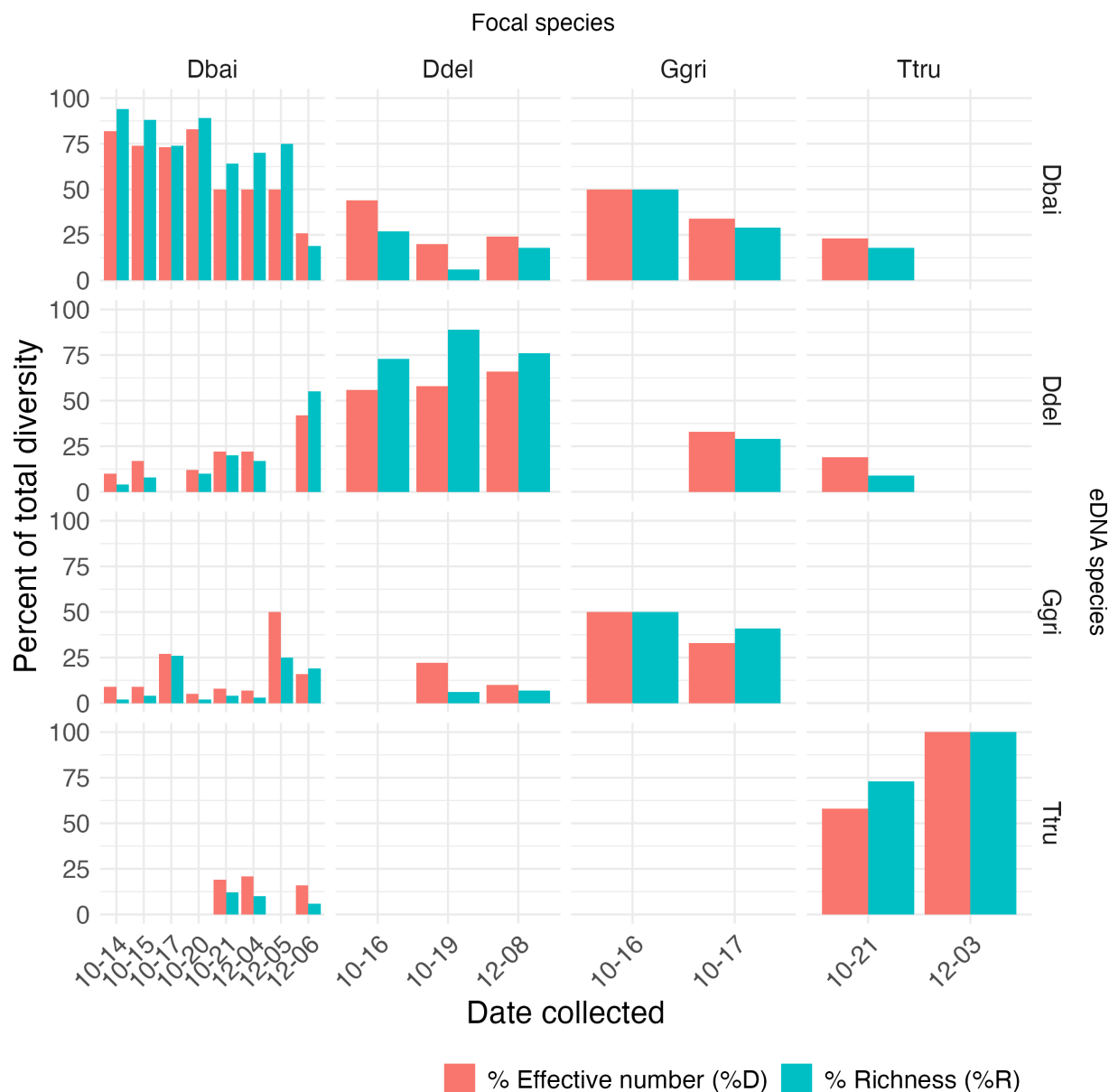


FIGURE 4

Percent of total diversity (y-axis) within encounters (x-axis) for each focal species (columns) and eDNA species (rows). Bars are color-coded to show percent of total effective number of ASVs (red), and ASV richness (teal) within focal species.

and Turner, 2016). Thus, we should not assume that the absolute amount of eDNA from each species is proportional to their relative biomass in the region without conducting detailed calibrations.

While these inter-specific differences could affect the total amount of DNA available in the water, they are unlikely to affect the relative ASV frequencies within each species. In every encounter, the focal species had the greatest ASV diversity (Figure 4). This suggests that a large proportion of the ASVs sampled were from dolphins within the focal school. In contrast, the presence of ASVs from non-focal species in all encounters is clear evidence that our sampling was also detecting the prior passage of other schools through the water mass. This is not surprising given that the four focal species are the most commonly occurring species in the region and occur around Catalina Island at high abundances and frequencies (Carretta et al., 2024). A consequence of this “background” eDNA signal is that we cannot assume that the

estimated genetic diversity of the focal species from an encounter directly represents the genetic diversity of the group being followed. If we were interested in better estimating the diversity of a specific school, we would have to collect water in the general area prior to the focal school’s arrival. We would then subtract this background diversity from the diversity of the focal follow itself.

We observed high variability of ASV occurrence and frequency among water samples, even within the same encounter. This sampling variability is well known in eDNA studies (Beentjes et al., 2019; Mathieu et al., 2020) and is the main reason for taking multiple samples to characterize diversity in other studies (Goldberg et al., 2016; Deiner et al., 2017; Zinger et al., 2019). Using previously published eDNA samples collected from lakes in Quebec, Canada, Yates et al. (2023) estimated that approximately 20 samples of 2L each (= 40 L) were required to sufficiently estimate the average overall eDNA

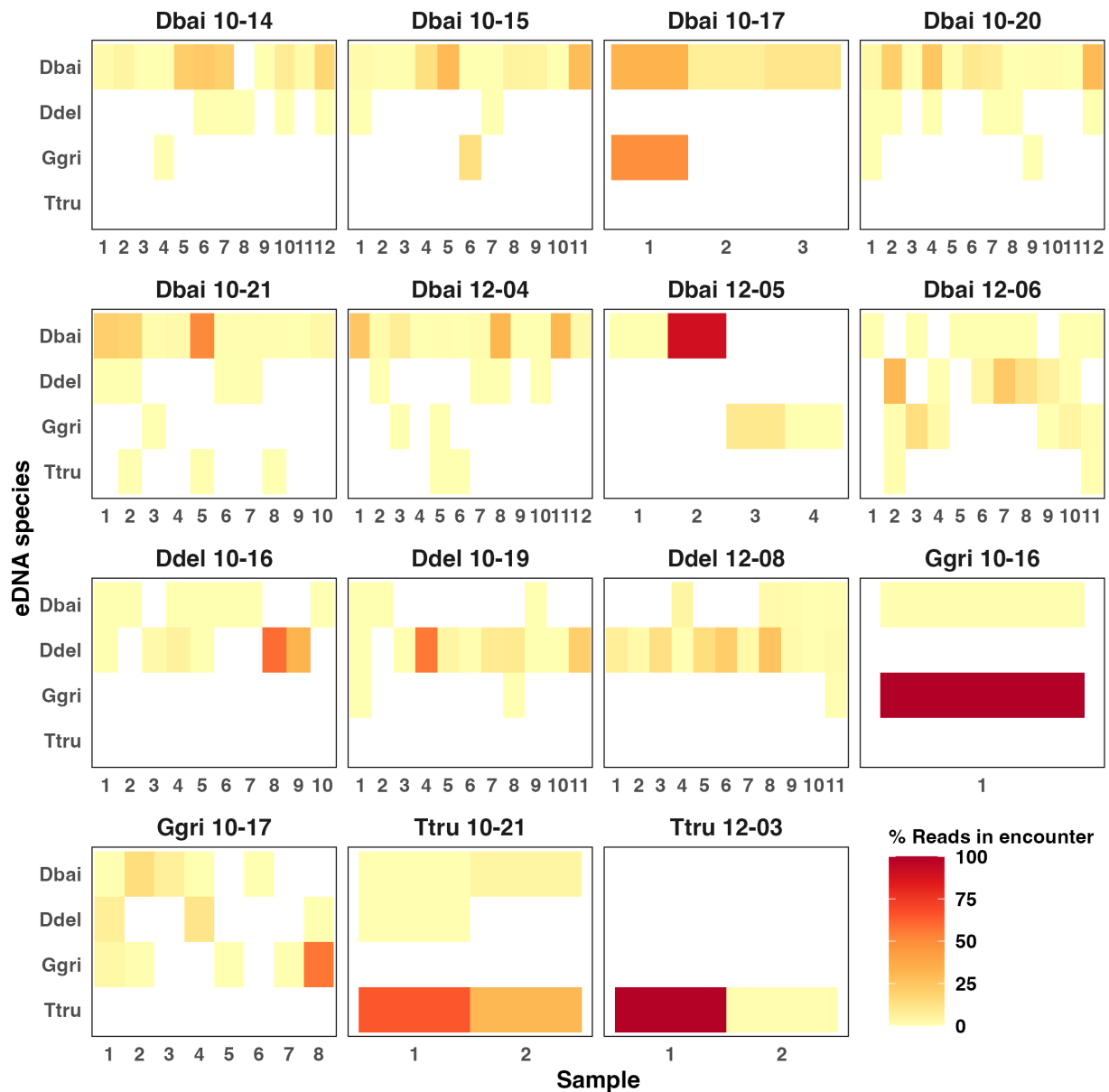


FIGURE 5

Percent of reads of each eDNA species (y-axis) in each encounter (box) by sample number within the encounter (x-axis). Labels above boxes for each encounter contain focal species of the encounter (Dbai = *Delphinus delphis bairdii*, Ddel = *D. d. delphis*, Ggri = *Grampus griseus*, and Ttru = *Tursiops truncatus*) and month and day of sample collection.

concentration within a lake. Cantera et al. (2019) estimated that 34 to 68 L was sufficient to estimate the species diversity of approximately 106 fish species in rivers and streams in French Guiana. Our resampling simulations similarly indicated that multiple encounters and samples within an encounter were required to begin to estimate true population genetic diversity, on the order of 60 to 72 L of water for long-beaked common dolphins. However, we stress that the number of eDNA samples required to assess the genetic diversity of an encounter is a function of the amount of overall genetic diversity in the wider population.

eDNA metabarcoding studies typically focus on assessing the community composition of particular taxa (Goldberg et al., 2016; Deiner et al., 2017; Adams et al., 2019). A concern in these studies is that the relative frequencies of ASVs may be influenced by

preferential PCR amplification of certain taxa due to greater similarity of the primers being used to some taxa rather than others. To mitigate this effect, it is recommended to create mock communities of some of the more frequent taxa with known concentrations to calibrate the empirical results (Ledger et al., 2024; Shelton et al., 2023; Skelton et al., 2023; Sickel et al., 2023). However, because we were interested in assessing diversity within each species rather than comparing the species' relative occurrence frequencies, we did not consider that any taxon-specific PCR biases would affect our analyses. Additionally, the PCR primers we used have been shown to be efficient for a wide range of delphinids, especially relatively closely related species like the four focal species in our study (Baker et al., 1996, 2018, 2023).

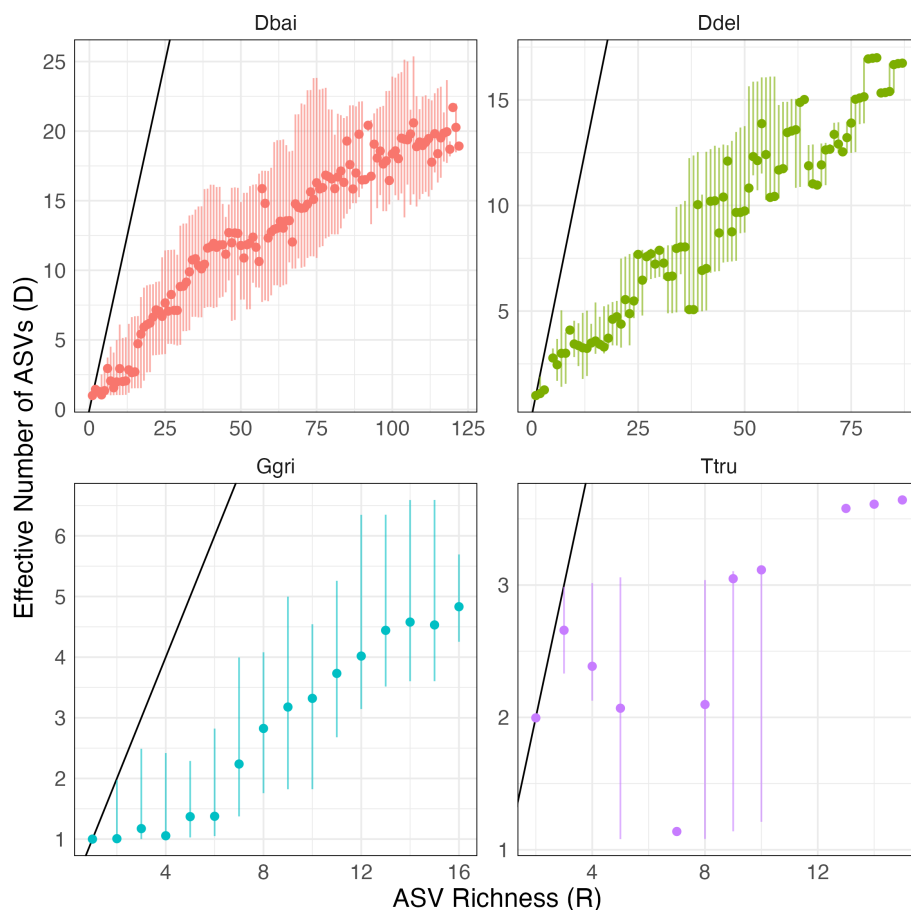


FIGURE 6

Distribution of effective number of ASVs (y-axis) for unique values of ASV richness (x-axis) by eDNA species (Dbai = *Delphinus delphis bairdii*, Ddel = *D. d. delphis*, Ggri = *Grampus griseus*, and Ttru = *Tursiops truncatus*). Points are median values from permutations, and bars indicate extent of central 95% HPDI. Solid black line indicates 1:1 slope.

Because our primary focus was characterizing within-species diversity, we had to develop a method for assigning ASVs to species with high confidence. The commonly used program, DADA2, assigns ASVs to species only if there is an exact, unambiguous match in the reference database (Callahan et al., 2016). As discussed previously, the large genetic diversity in some of the focal species, made it unlikely that our reference databases would be sufficiently complete for DADA2 to assign the number of new ASVs expected in the eDNA samples. Additionally, although there are strong frequency differences between the mtDNA Control Region of the common dolphins, there are no diagnostic sites for distinguishing the two species (Jefferson and Archer, 2024). Thus, it would be difficult for DADA2 to assign some *Delphinus* ASVs unambiguously, especially if they are shared between the taxa. Our method uses a combination of BLAST sequence matching and a Random Forest classification model to both assign these ASVs as well as to estimate the uncertainty of each assignment. The Random Forest model harnesses the frequency differences of substitutions in the reference alignments to improve assignments over simple similarity measures used by BLAST (Archer et al., 2017). Finally, our pipeline minimizes the inclusion of erroneous ASVs by examining the pattern of substitutions for ASVs that are not assigned by a perfect match, a feature that is also unavailable in DADA2.

Our four focal species are understood to exist in what has been termed “fission-fusion” societies, where individuals form schools that split and reform with new individuals on a regular basis (Perryman and Lynn, 1993; Evans, 1994; Connor et al., 2000). Common dolphins are also well known for occurring in very large schools, sometimes ranging into the thousands (Evans, 1994). On the other hand, Risso’s and bottlenose dolphin group sizes tend to be much smaller. There is also no evidence of multiple populations of common dolphins or Risso’s dolphins along the US west coast. Thus, we consider the ASV frequency distributions within each of these four species to be a random sample of the true population genetic diversity.

eDNA is rapidly becoming an important tool for monitoring cetacean populations (Alter et al., 2022; Afonso et al., 2024; Parsons et al., 2025; Deng et al., 2025; Bennington et al., 2024). While it is unclear to what extent eDNA surveys will replace traditional methods, our study demonstrates its utility for efficiently assessing and comparing genetic diversity in social odontocetes. The fact that we were able to conduct this study on some of the most abundant species of dolphins indicates that our methods are likely to be applicable to most cetacean taxa. Our hope is that our methods and results prove useful to help guide the development of sampling protocols in future studies as this field matures.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by National Marine Fisheries Service Permits 1435, 19116, 21678. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

FA: Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. DS: Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. BS: Investigation, Writing – review & editing. SF: Investigation, Writing – review & editing. JC: Investigation, Writing – review & editing. JD: Investigation, Writing – review & editing. HF: Investigation, Writing – review & editing. CB: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

Authors BS and SF were employed by Southall Environmental Associates.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CR declared a past co-authorship with the author JC to the handling editor.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2026.1756593/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Workflow for assignment of ASVs to species.

SUPPLEMENTARY FIGURE 2

Distribution of effective number of ASVs (y-axis) from permutations of different numbers of samples within encounters of each focal species (columns) for ASVs of each eDNA species (rows). Data for bottlenose dolphins (Ttru) have been excluded due to low sample sizes. Red line shows total effective number of ASVs across all samples and encounters.

SUPPLEMENTARY FIGURE 3

Percent of permutations replicates of encounters and number of samples with ASV richness equal to or greater than the overall richness for each focal species (columns) and eDNA species (rows).

SUPPLEMENTARY FIGURE 4

Percent of permutations replicates of encounters and number of samples with effective number of ASVs equal to or greater than the overall effective number of ASVs for each focal species (columns) and eDNA species (rows).

SUPPLEMENTARY TABLE 1

Summary of ASV species assignment pipeline. Shown are the step numbers as given in the text (Step), a description of the step (Description), total number of reads (Reads), number of reads per run (Reads/Run), number of ASVs retained at the end of the step (ASVs), percent of reads retained from the beginning (% Reads), and percent of ASVs retained from the beginning (% ASVs).

SUPPLEMENTARY TABLE 2

Spreadsheet of Genbank accession numbers and species used in mtDNA reference alignments.

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