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Abstract

Restriction site-Associated DNA tag (RAD-tag) sequencing has become a popular approach to generate thousands of SNPs used to address diverse questions in population genomics. Comparatively, the suitability of RAD-tag genotyping to address evolutionary questions across divergent species has been the subject of only a few recent studies. Here, we evaluate the applicability of this approach to conduct genome-wide scans for polymorphisms across two cetacean species belonging to distinct families: the short-beaked common dolphin (*Delphinus delphis*; n = 5 individuals) and the harbor porpoise (*Phocoena phocoena*; n = 1 individual). Additionally, we explore the effects of varying two parameters in the `Stacks` analysis pipeline on the number of loci and level of divergence obtained. We observed a 34% drop in the total number of loci that were present in all individuals when analyzing individuals from the distinct families compared to analyses restricted to intra-specific comparisons (i.e., within *D. delphis*). Despite relatively stringent quality filters, 3,595 polymorphic loci were retrieved from our inter-familial comparison. Cetaceans have undergone rapid diversification and the estimated divergence time between the two families is relatively recent (14 to 19 My). Thus, our results showed that, for this level of divergence, a large number of orthologous loci can still be genotyped using this approach, which is on par with two recent *in silico* studies. Our findings constitute one of the first empirical investigations using RAD-tag sequencing at this level of divergence and highlights the great potential of this approach in comparative studies and to address evolutionary questions.

Introduction

Recent parallel DNA sequencing technologies have enabled population genomics studies in non-model organisms including characterizing patterns of hybridization and introgression (e.g. Hohenlohe *et al.* 2011), intra-specific phylogeography (e.g. Emerson *et al.* 2010), QTL mapping (e.g. Gagnaire *et al.* 2013) and studying the genetic basis of adaptations (Stapley *et al.*, 2010). There is now a growing interest in these methods in the fields of biogeography (Lexer *et al.*, 2013) and phylogenetics (McCormack *et al.*, 2013).

Among recent genotyping methods using next-generation sequencing, Restriction site-Associated DNA tag (RAD-tag) sequencing has become one of the most popular approaches to conduct population genomics studies in non-model organisms. To date, however, few studies have explored the applicability of this approach to divergent species to address evolutionary questions at a greater phylogenetic depth. Two *in silico* studies evaluated the suitability of RAD-tag sequencing to address phylogenetic questions (Rubin *et al.* 2012; Cariou *et al.* 2013) using simulated datasets obtained from divergent reference genomes. Rubin *et al.* (2012) used genomes from three taxonomic groups (*Drosophila*, mammals and yeasts) to generate RAD-tag sequences *in silico* and, for each group, assessed whether accurate species phylogenies could be reconstructed from these sequences. Similarly, Cariou *et al.* (2013) simulated RAD-tag sequences from the genomes of 12 species of *Drosophila*, separated by different levels of divergence (5 to 63 Mya). Both studies suggest that 1) a sufficient number (at least hundreds) of conserved orthologous loci can be obtained even when comparing divergent species within relatively young phylogenetic groups (divergence times of up to 60 Mya), and 2) RAD-tag loci can be phylogenetically informative and allow reconstruction of accurate species phylogenies.

Few empirical studies have evaluated whether these expectations are verified by including divergent species in their RAD-taq sequencing analysis (Eaton & Ree 2013; Nadeau *et al.* 2013; Stölting *et al.* 2013), and particularly beyond intra-generic (Keller *et al.*, 2012; Jones *et al.*, 2013; Lexer *et al.*, 2013; Wagner *et al.* 2013) or intra-familial (Bergey *et al.*, 2013) comparisons. In the present study, we assessed

the applicability of RAD-tag genotyping in the upper bound of these phylogenetic depths by conducting intra- and inter-familial comparisons using two cetacean species: the common dolphin (*Delphinus delphis*, Delphinidae) and the harbor porpoise (*Phocoena phocoena*, Phocoenidae). We analyzed generated RAD-tag sequences using the `Stacks` analysis pipeline (Catchen *et al.*, 2011) and evaluated the effects of varying two `Stacks` parameters on the number of loci and genetic distances obtained.

Materials and Methods

Tissue samples, DNA extraction and Sanger sequencing

Tissue samples were collected from six dead animals (five short-beaked common dolphins, *Delphinus delphis*, and one harbor porpoise, *Phocoena phocoena*) that were either incidentally caught in pelagic fisheries in the Celtic Sea or Bay of Biscay, or stranded on the French Atlantic coast (Table 1). Tissue samples were frozen at $-20°$C or stored in ethanol at room temperature. Total genomic DNA was extracted from approximately 15 to 25 mg of skin or kidney tissue using NucleoSpin® Tissue (Macherey-Nagel EURL, Hoerdt, France) or using DNeasy® Blood & Tissue (Qiagen, Courtaboeuf, France) kits following the manufacturer's protocols. DNA concentration was quantified using a NanoDrop™ 2000 (Thermo Scientific, Illkirch, France). DNA quality was assessed on a 1% agarose gel stained with ethidium bromide and was similar across the six samples: good (high molecular weight as well as shear) to excellent (high molecular weight only). Species identification made in the field using morphological characters was confirmed by sequencing two portions of the mitochondrial genome: 1) the 5' end of the control region (including a portion of the flanking proline tRNA) was amplified using primers L15824 (5'-CCTCACTCCTCCCTAAGACT-3'; Rosel *et al.* 1999) and H16498 (5'-CCTGAAGTAAGAACCAGATG'-3; Rosel *et al.* 1994); 2) a portion of cytochrome *b* was amplified using primers L14724 (5'-TGACTTGAARAACCAYCGTTG-3'; Palumbi *et al.* 1991) and H15149 (5'-CAGAATGATATTTGTCCTCA-3'; Kocher *et al.* 1989). The polymerase chain reaction (PCR) included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 0.3 μM of each primer, 0.15 mM dNTPs (Euromedex, Mundolsheim, France), 2 U Taq poly-

merase (VWR, Fontenay sous Bois, France), and 50 ng DNA in a 50 μL total volume. PCR profiles were as described in Vollmer *et al.* (2011) for the L15824/H16498 primer pair and Viricel & Rosel (2012) for the L14724/H15149 primer pair. PCR products were sent to Genoscreen (Lilles, France) for purification and Sanger sequencing. Mitochondrial sequences were edited using Sequencher® v. 4.7 (Gene Codes Corp., Ann Arbor, MI, USA) and were aligned using `MAFFT` v. 7 with default parameters (FFT-NS-i method) (Katoh *et al.*, 2002).

Genotyping by sequencing

RAD-tag libraries were prepared by Eurofins Genomics (Ebersberg, Germany) using 1-2 μg of total genomic DNA per individual and using the *Not1* restriction enzyme. Unique barcodes used to differentiate multiplexed individuals were six to nine nucleotides long and differed by at least two nucleotides. Libraries were sequenced by Eurofins Genomics on two lanes of the Illumina® HiSeq™ 2000 platform (Illumina, Inc., San Diego, CA, USA) with the 1 x 100 base-pairs (bp) single-end read module, as part of a larger *D. delphis* population genomics RAD-tag sequencing project (total of 92 individuals). Raw Illumina reads were processed using the `CASAVA` v. 1.8.2 software (Illumina, Inc., San Diego, CA, USA). Illumina read data were de-multiplexed, quality-filtered and assembled using the `Stacks` tool kit v. 0.99994. A recent study by Davey *et al.* (2013) compared `Stacks` and `RADtools` (Baxter *et al.*, 2011), another program to analyze RAD-tag sequences without a reference genome, and recommended use of `Stacks` as it provides more features. The `Stacks` pipeline includes four major steps (Catchen *et al.*, 2013): reads are first sorted by unique barcode to group together all sequences from each individual (de-multiplexing step) while also excluding sequences that do not pass a set quality score; second, loci are build within each individual by creating stacks of identical reads and assembling unique loci by merging stacks that differ only by a set number of nucleotides (M) to allow polymorphism within individuals; third, loci identified for each individual are compared and catalogued across all individuals and a set number of nucleotide differences (n) is allowed to merge loci from different individuals in the catalog; fourth, individual genotypes are determined for each locus. The following filters were applied during the first step

of the pipeline (`process_radtags`): one nucleotide mismatch (i.e. one sequencing error) was tolerated within individual barcodes, sequences for which the mean raw Phred quality score dropped below 10 within a sliding window spanning 15% of the read length were discarded. Sequences were truncated to a length of 91 bp. Since there is no reference genome available for *D. delphis* or *P. phocoena*, we used the `denovo_map.pl` program in `Stacks` to merge stacks (*i.e.*, sets of identical reads) into loci within individuals and to build a catalog of loci across individuals. The minimum number of reads to form a stack (m) was set to 3. SNPs were detected while varying two `Stacks` parameters: the number of mismatches allowed between stacks to be grouped in a unique locus within an individual (M, set between 1 and 7), and the maximum distance among loci from distinct individuals to be merged in the population catalog (n, set between 1 and 8). Analyses were conducted with 14 different combinations of these parameters setting M and n at the same value or with one additional mismatch for n compared to M (see Figures 1 and 2). Highly repetitive sequences were removed or broken down using the 't' option in `denovo_map.pl` (Catchen *et al.*, 2011, 2013). Additionally, we verified that the final catalog did not contain dimers formed by adapters. The quality of filtered sequences obtained after `denovo_map.pl` was evaluated using `FastQC` v. 0.10.1 (Babraham Bioinformatics, `www.bioinformatics.babraham.ac.uk/projects/`). We applied the `populations` program from `Stacks` to obtain the final sets of orthologous loci: loci were retained if the locus total depth of coverage was equal or higher than 10 reads per individual, and if they were present in all individuals (i.e. no missing data allowed).

Data analyses

Polymorphism and divergence statistics were calculated using the `populations` program in `Stacks`, and using the `ape` (Paradis *et al.*, 2004) and `adegenet` (Jombart, 2008) packages in `R` (R Development Core Team, 2013), respectively. Inter-individual divergence was assessed using polymorphic sites that are either variable within individuals (in heterozygotes), or fixed within individuals (homozygotes) but variable between individuals. Due to likely heterogeneity in substitution models across loci, genetic distances were calculated as raw p-distances (i.e., proportion of fixed differences between two sequences).

129 To compare intra-specific and inter-familial data, all analyses were run on: 1) the five *Delphinus delphis*
130 individuals, and 2) all six individuals (five *D. delphis* and one *Phocoena phocoena*).

131 Finally, for the inter-familial comparison, we explored the functions of invariable (conserved) compared
132 to polymorphic loci, at a chosen M and n combination (M3n3; see Results section). The goal of this
133 analysis was two-tiered: 1) to investigate whether some functions would be overrepresented in polymorphic
134 compared to invariable loci, 2) to assess whether putative gene functions can be retrieved from RAD-
135 tag sequences, which could be useful in applications such as studying loci potentially under selection.
136 Identification of RAD tag sequences (1,587 and 3,574 tags for conserved and variable loci, respectively)
137 was determined in `Blast2GO` v. 2.6.6 (public database of August 2012; Conesa *et al.*, 2005; Conesa &
138 Götz, 2008; Götz *et al.*, 2011, 2008) using the `blastn` program (e-value cut off of 10^{-3}, HSP cut-off of 33
139 Altschul *et al.*, 1990, 1997), as the `blastx` program retrieved very little results due to the short length of
140 the corresponding amino-acid sequences (< 31 amino-acids). While `blastn` can be used to identify tags,
141 it does not allow subsequent mapping and annotation (`Blast2GO` manual). We therefore used the results
142 of `blastn` to retrieve the sequence of the best match between tags and Genbank sequences from the
143 *Tursiops truncatus* genome (Genbank Bioprojects accession numbers PRJNA189944 and PRJNA20367),
144 a species closely related to *D. delphis* (McGowen *et al.*, 2009), similar to the approach employed by
145 Reitzel *et al.* (2013) for the anemone *Nematostella vectensis*. As the percent identity between tags and *T.*
146 *truncatus* sequences was very high (84.6 to 100%), we proceeded to the mapping and annotation steps to
147 obtain Gene Ontology (GO) terms from these longer *T. truncatus* sequences (222 to 16,700 bp, median
148 size 1,549 bp; `blastx` e-value cutoff = 10^{-3} HSP = 33, 20 hits retained; annotation settings: e-value filter
149 = 10^{-6}, annotation cutoff = 55, GO weight = 5, no HSP-hit coverage cutoff). GO terms correspond to
150 groups of genes involved in similar functions such as genes with products involved in cellular components.
151 Genes can be grouped into GO terms at different levels depending on the desired level of precision in the
152 function. Enrichment of GO terms between *T. truncatus* sequence sets corresponding to "conserved" and
153 "variable" loci were tested using the Fisher's Exact test as implemented in `Blast2GO` (GOSSIP module,

Blüthgen *et al.*, 2005, FDR=0.05).

Results

Species identification of each individual was confirmed using Sanger-sequenced mitochondrial DNA. Mitochondrial sequence alignments encompassed a 425 bp portion of the control region and a 402 bp portion of cytochrome *b* (see Table 1 for Genbank accession numbers). For the five common dolphins, control region sequences were identical to haplotypes published on Genbank (i.e. 100% coverage and 100% identity), which were sequenced from other short-beaked common dolphins from the eastern North Atlantic (NA). Cytochrome *b* sequences for these individuals also supported species identification made in the field as most similar sequences in Genbank belonged to short-beaked common dolphins. For the harbor porpoise, we obtained a perfect haplotype match for the mitochondrial control region sequence, corresponding to another harbor porpoise from the eastern NA. For cytochrome *b*, there was a one bp difference between our sequence and a published haplotype (accession number: AJ554063) from a harbor porpoise complete mitogenome. The next best match in Genbank was also a harbor porpoise (accession number: U13143).

The two Illumina sequencing lanes produced over 2.8 million raw reads per individual (Table 2). On average, 40% of raw reads were removed by the quality filters applied (Table 2). The main reason for removing reads was ambiguous barcodes, which could suggest either barcode synthesis errors, or a high sequencing error rate. By setting the minimum number of reads to build a stack to 3, the impact of potential sequencing errors on the genotypes and loci we obtained should be very limited. The sequence quality of filtered reads was excellent with a minimum Phred score of 35 (Table 2).

The effect of varying the `denovo_map.pl` parameters M and n can be contrasted between the intra-specific and inter-familial datasets. For the inter-familial comparison, increasing M (intra-individual parameter), and particularly n (inter-individual parameter) resulted in an increase in the total number of loci until a plateau was reached at parameter combination M3n3 (Figure 1a; total number of loci: 5182). This outcome can be explained as follows: in the first step of the analysis (*i.e.*, `denovo_map.pl`),

increasing M and n will decrease the total number of loci present in the catalog as more distinct sequences will be merged into the same locus (Catchen et al. 2013). This also results in a greater depth of coverage per locus. Thus, in the second step of the analysis (`populations`), there will actually be an increase in the total number of loci that are kept in the final catalog after the filters are applied (a minimum of 10 reads per locus per individual). Additionally, as n is increased, more loci will be in common among all individuals, particularly when including divergent individuals such as here. When n is low, fixed differences will be considered as distinct loci that will not be present in all individuals. Therefore, the number of loci kept in the final catalog will also increase when n is increased due to the filter of the minimum number of individuals where a locus has to be present (in this study, a locus had to be present in all individuals). A similar trend can be observed for the number of polymorphic loci, which increased as M and n increased (Figure 1b). Eventually, increasing M and n could result in overmerging loci (loci that are not orthologous). By using the set of parameters corresponding to where a plateau for the total number of loci starts, we were most likely to avoid overmerging issues. However, it is possible that overmerging is not detectable by simply observing a plateau in the total number of loci, as the overall decrease in the number of loci by overmerging could be balanced by the discovery of orthologous, yet highly divergent loci. For the intra-specific dataset, the effect of varying the two parameters on the number of loci (total and polymorphic) was less striking (Figures 1a,b). A plateau was quickly reached at the M2n2 parameter combination (total number of loci: 7838; 2032 polymophic loci) after an initial small rise in the number of polymorphic loci (Figure 1b). In terms of divergence, the largest change for both datasets was observed when increasing n from 1 to 2 (Figure 2a,b). This is likely due to an increase in the number of variable sites that are fixed within but variable among individuals. For subsequent data description (e.g. sequence variability) and analyses (`Blast2GO`), we chose the parameter combinations where a plateau was reached in terms of number of loci, which corresponded to M3n3 for the inter-familial dataset and M2n2 for the intra-specific dataset.

Using these parameter combinations, we observed a 34% drop in the total number of loci when

analyzing all individuals (in the inter-familial comparison) compared to the intra-specific dataset. This drop was calculated as the percent difference in the total number of loci found with and without the *P. phocoena* sample in the final dataset. This result was not simply an effect of removing any individual from the dataset, as excluding a *D. delphis* individual only resulted in a small percent difference (2.2 to 6.5 %) in the total number of loci. Thus, we can conclude that most of the 34% percent drop in number of loci was indeed due to including a more divergent individual. The proportion of variable loci was 69% and 26% for the inter-familial and intra-specific datasets, respectively. In terms of sequence polymorphism, we observed one SNP every 292 bp in the intra-specific dataset (total sequence length screened: 713,255 bp) compared to one SNP every 71 bp in the inter-familial dataset (total sequence length screened: 471,538 bp).

In the inter-familial comparison, sequence identification using `Blast2GO` was significantly higher for conserved tags (994/1587, 63%) than for polymorphic tags (1512/3574, 42%). `blastn` searches were reliable, with e-values ranging from 10^{-5} to 10^{-38} for the best hit. Similarity between query and subject sequences of the best hit was high (73-100%, median 100% for conserved tags; 71-100%, median 98% for polymorphic tags). Top species hits included sequences from killer whale (*Orcinus orca*: 774 hits), common bottlenose dolphin (*Tursiops truncatus*: 716 hits), human (*Homo sapiens*: 234 hits), and other mammals.

A majority of the best hit sequences from *T. truncatus* could be mapped and annotated (conserved: 91%; variable: 92%). There was no significant difference in the number of *T. truncatus* sequences per GO term between variable versus conserved loci across the two cetacean families (Fisher's exact test with FDR = 0.05; Figure S1, supporting information).

Discussion

Modern cetaceans constitute a recent group, which originated approximately 34 to 35 mya (Fordyce, 1980; Arnason *et al.*, 2004) and comprise 14 extant families (Perrin *et al.*, 2009). Thus, based on the

two *in silico* studies from Rubin *et al.* (2012) and Cariou *et al.* (2013), a large number of conserved orthologous loci should be obtained when comparing species from distinct cetacean families. Indeed, our study confirms this expectation, as only 34% of the loci present, at this sequencing effort, in all common dolphins were lost when including an individual from a distinct cetacean family. The divergence time separating these two families (Delphinidae and Phocoenidae) has been estimated between 14 and 19 Mya based on fossil-calibrated molecular clocks (Arnason *et al.*, 2004; Xiong *et al.*, 2009; McGowen *et al.*, 2009). Comparatively, the proportion of loci that were lost (34%) in our inter-familial comparison was lower than the percentage of loci lost (60%) between two divergent *Drosophila* species pairs, which have been separated for a similar period of time (ca 13 My) (Cariou *et al.*, 2013). At similar divergence times, the loss of orthologous loci will depend on the rate of molecular evolution, which varies among taxonomic groups (Britten, 1986; Martin & Palumbi, 1993). Indeed, *Drosophila* has a high nucleotide substitution rate (e.g. Britten, 1986; Chan *et al.*, 2012) compared to cetaceans, which generally display slow rates of molecular evolution (Kingston & Rosel, 2004; Bininda-Emonds, 2007; McGowen *et al.*, 2012).

We explored the effects of varying two parameters in the `denovo_map.pl` program of `Stacks` on the number of loci and level of divergence obtained. A plateau was reached, after which the number of loci did not change dramatically. Our results are comparable to Keller *et al.* (2012) who observed a decrease in the total number of loci obtained when increasing M and n, prior to applying filters, and an increase in the number of polymorphic loci after filters were applied. The level of sequence variability we observed in intra-specific comparisons (within *D. delphis*: one SNP every 292 bp) was comparable to the diversity previously observed in *Delphinus* spp. or in closely related species. Thus, Amaral *et al.* (2010) screened 6,537 bp in 17 *Delphinus* spp. individuals and reported a SNP every 272 bp. For the common bottlenose dolphin (*Tursiops truncatus*), Vollmer & Rosel (2012) observed one SNP every 463 bp (total sequence length screened: 70,828 bp in 10 individuals). Note that the five common dolphins analyzed here were from the eastern NA, and possibly from the same population. Greater sequence variability would be expected if individuals from distinct regions or populations were analyzed as in Vollmer & Rosel (2012)

and Amaral *et al.* (2010). A source of sequence variability that we did not consider here is the occurrence of indels. `Stacks` does not allow for indels and these sequences would appear as distinct loci in our analysis and would not pass the filter of being present in all individuals. Therefore, we may have lost some loci if indels were present. Among alternative pipelines that have been developed to analyze RAD-tag data in absence of a reference genome, `PyRAD` (Eaton & Ree, 2013) does accommodate indels. `PyRAD` is based on sequence similarity and alignment, rather than a set number of nucleotide differences.

The number of polymorphic loci and sequence variability (one SNP every 71 bp) observed in our inter-familial comparison outline the potential benefits of RAD-tag sequencing to solve phylogenetic questions within a group that diversified in multiple rapid and recent radiation events (Steeman *et al.*, 2009). Recently, analysis of amplified fragment length polymorphisms (AFLPs) has provided new insights into the phylogeny of cetaceans (Kingston & Rosel, 2004; Kingston *et al.*, 2009). However, these markers are dominant and anonymous. One advantage of RAD-tag sequencing compared to the approach above is that it provides co-dominant sequence data, which can be potentially identified and annotated using published databases (e.g. Scaglione *et al.*, 2012). Our `Blast2GO` results suggest that insights into putative function can be gained by comparing short (<100 bp) RAD-tag sequences to published sequences. However, GO terms associated with RAD sequences could not be obtained directly using `blastx` due to the short length of the corresponding amino-acid sequences and `Blast2GO` does not produce GO terms when `blastn` is applied. Thus, obtaining GO terms was achieved indirectly by relying on `blastn` hits from the reference genome of a closely related species (*T. truncatus*) and applying `blastx` on these longer sequences. While we limited our phylogenetic analyses to comparing GO terms between conserved and polymophic loci, and calculating genetic distances, `Stacks` produces outputs that allow to conduct other analyses widely-used in phylogeny or phylogeography such as building phylogenetic trees and running cluster analyses.

One limitation of RAD-tag sequencing for phylogenetic inferences is that the number of loci is expected to decrease as more taxa are added to the dataset (as seen in Lexer *et al.*, 2013), which will limit to some extent the size of the phylogenetic dataset. Thus, there will be a trade-off between the number of taxa and

277 the total number of orthologous loci analyzed. One way to alleviate this issue could be to use a RAD-
278 tag double-digest approach (Peterson *et al.*, 2012), which would increase locus representation across
279 individuals. Very recently, new high-throughput genomic sequencing approaches have been developed
280 to specifically target phylogenomics and phylogeographic questions (Carstens *et al.*, 2012; Lemmon &
281 Lemmon, 2013). These new laboratory methods should be complementary to applications of RADseq
282 data. A first approach, developed by Lemmon *et al.* (2012), is based on a sequence capture technique,
283 which relies on probes designed using sequenced reference genomes. This approach, termed *anchored*
284 *enrichment*, can provide several hundreds of loci, in the form of sequence data, for potentially hundreds
285 of individuals from model and non-model organisms and should be applicable at different phylogenetic
286 depths. An advantage of this approach, is that it should be applicable to degraded samples. To date
287 however, its applicability to recently and rapidly evolved groups has not been empirically assessed yet.
288 A second approach was designed to investigate relationships at greater phylogenetic depths by targeting
289 ultraconserved elements (Faircloth *et al.*, 2012; McCormack *et al.*, 2012). While RAD-tag sequencing
290 may be more appropriate for questions related to species delimitation and phylogeography in rapidly
291 and recently diverged groups (e.g., cetaceans), other approaches such as *anchored enrichment* should be
292 used when the phylogenetic depth in question reaches the limits of utility of RAD-tag data (for a full
293 comparison of these methods, see review by Lemmon & Lemmon, 2013). In the near future, we should
294 gain a better sense of the limits of each approach as studies implementing these new methods accumulate.
295 In conclusion, our empirical study supports expectations that the applicability of RAD-tag genotyping
296 is not limited to closely related species. Using two mammalian species from distinct, but recently evolved,
297 families, we showed that this approach holds great promise for evolutionary studies conducted at this
298 phylogenetic level.

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Data Accessibility

Mitochondrial DNA sequences have been submitted to Genbank (see Table 1 for accession numbers). Demultiplexed and filtered (i.e. after process-radtags) sequences (.fq files), `R` and `Stacks` codes, and `Blast2GO` output files were deposited in Dryad (doi:10.5061/dryad.mk364).

Author Contributions

A.V. and B.S. designed the research; W.D. provided tissue samples and voucher information; A.V. conducted laboratory work; A.V. and E.P analyzed the data; A.V. and E.P wrote the manuscript.

Figure Legends

Fig. 1 Influence of Stacks parameters on (a) the total number of loci, and (b) the number of polymorphic loci obtained. Fourteen parameter combinations were evaluated for the whole dataset (inter-familial comparison), and for the common dolphin only (intra-specific comparison).

Fig. 2 Influence of Stacks parameters on inter-individual sequence divergence (raw p-distances calculated using variable sites) for (a) inter-familial comparisons, and (b) intra-specific comparisons. The range of inter-individual distances is represented as boxplots.

Table 1 Voucher information for one harbor porpoise (*Phocoena phocoena*) and five short-beaked common dolphins (*Delphinus delphis*) used in this study. Voucher identification (ID) numbers correspond to specimen numbers from UMS Pelagis. For samples obtained from stranded animals, the geographic coordinates and date of the stranding event are given. For one common dolphin that was incidentally caught (bycatch) in the tuna fishery, geographic coordinates and date correspond to where and when the dead animal was retrieved from the gear onboard. Genbank accession numbers are given for each mitochondrial DNA portion that was sequenced: control region (CR) and cytochrome *b* (*cytb*).

Species	Voucher ID	Sample type	Sex	Latitude	Longitude	Date	CR accession no	*cytb* accession no
Phocoena phocoena	10712131	bycatch	M	44.648	-1.316	13-Apr-07	KF727592	KF727598
Delphinus delphis	10307073	stranding	F	46.713	-1.979	29-Jul-03	KF727593	KF727599
Delphinus delphis	10401011	stranding	F	44.404	-1.264	17-Jan-04	KF727594	KF727600
Delphinus delphis	10512077	bycatch	F	48.100	-9.867	3-Sep-05	KF727595	KF727601
Delphinus delphis	9902012	stranding	M	43.955	-1.363	19-Feb-99	KF727596	KF727602
Delphinus delphis	10201010	stranding	F	46.189	-1.429	22-Jan-02	KF727597	KF727603

Table 2 Total number of raw and filtered (i.e. after process-radtags) reads for each sample used in this study. Overall sequence quality was assessed using the mean Phred score after filters from process-radtags .

Species	Voucher ID	Raw reads	Filtered reads	Mean Phred score
Phocoena phocoena	10712131	3,102,559	2,455,577	36
Delphinus delphis	10307073	3,125,400	1,614,204	36
Delphinus delphis	10401011	3,185,527	1,846,015	36
Delphinus delphis	10512077	2,814,062	2,091,276	36
Delphinus delphis	9902012	3,859,005	1,466,233	36
Delphinus delphis	10201010	3,712,290	2,105,664	35

Fig. 1

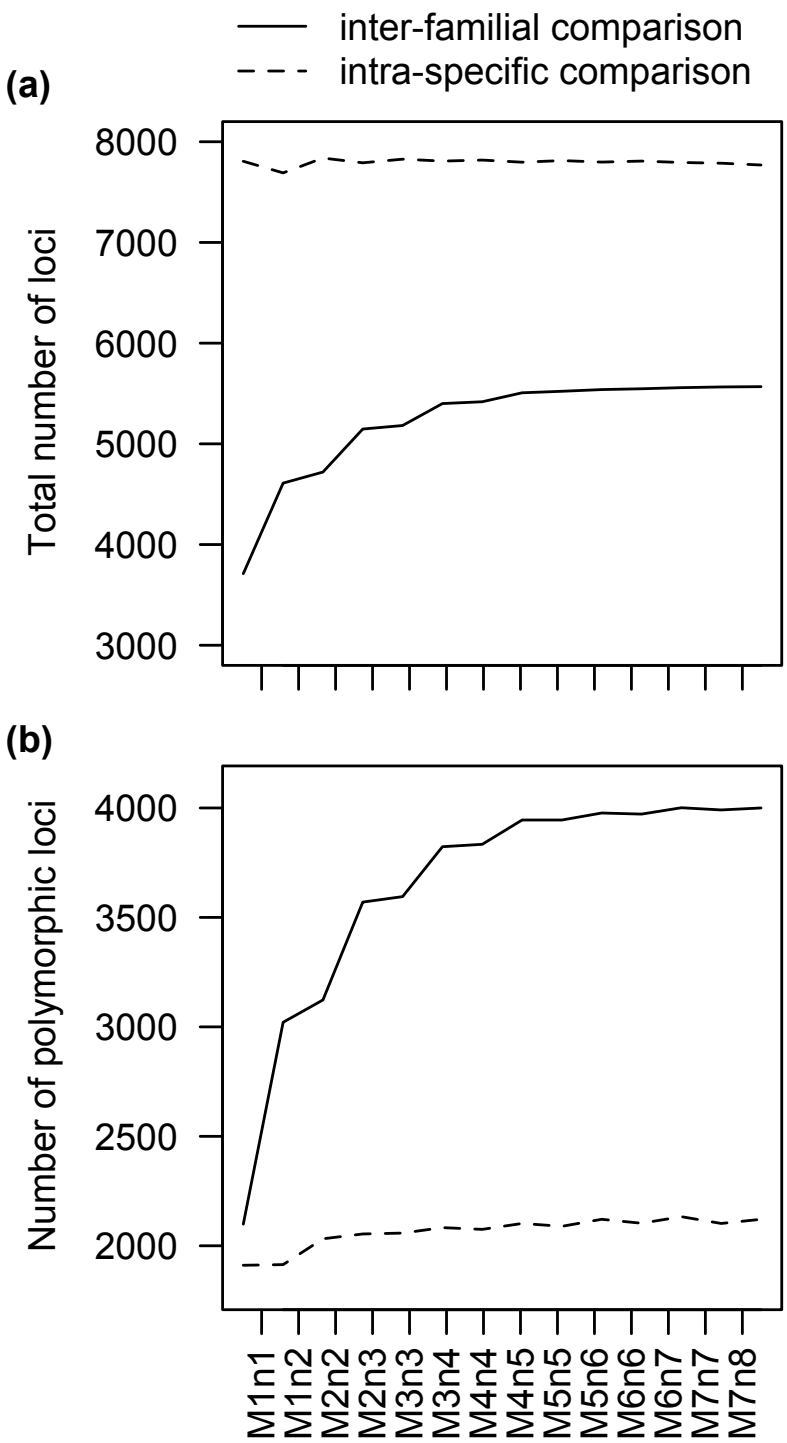
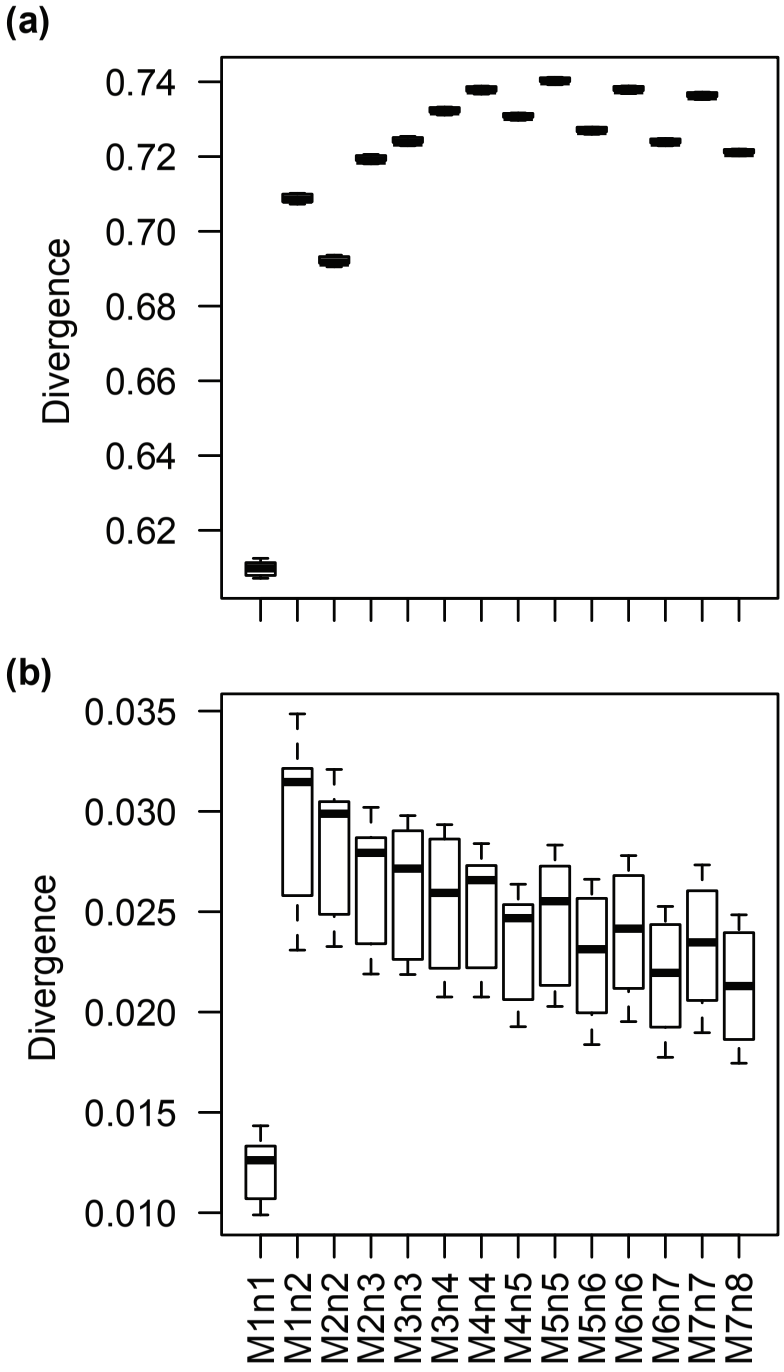

inter-familial comparison
intra-specific comparison
(a)
Total number of loci
8000
7000
6000
5000
4000
3000
(b)
Number of polymorphic loci
4000
3500
3000
2500
2000
M1n1
M1n2
M2n2
M2n3
M3n3
M3n4
M4n4
M4n5
M5n5
M5n6
M6n6
M6n7
M7n7
M7n8

Fig. 2


(a)
Divergence
0.74
0.72
0.70
0.68
0.66
0.64
0.62
(b)
Divergence
0.035
0.030
0.025
0.020
0.015
0.010
M1n1
M1n2
M2n2
M2n3
M3n3
M3n4
M4n4
M4n5
M5n5
M5n6
M6n6
M6n7
M7n7
M7n8