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RESEARCH ARTICLE

# Molecular identification of small cetacean samples from Peruvian fish markets

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**Abstract** In the last 60 years, incidental entanglement in fishing gears (so called by-catch) became the main cause of mortality worldwide for small cetaceans and is pushing several populations and species to the verge of extinction. Thus, monitoring and quantifying by-catches is an important step towards proper and sustainable management of cetacean populations. Continuous studies indicated that by-catches and directed takes of small cetaceans in Peru greatly increased since 1985. Legal measures banning cetacean takes, enforced in 1994 and 1996, ironically made monitoring highly problematic as fishers continue catching these animals but utilize or dispose of carcasses clandestinely. Hence, in locations where cetaceans are landed covertly or already butchered, molecular genetic methods can provide the only means of identification of the species, sex, and sometimes the population of each sample. Here, we generate and analyse a fragment of the mitochondrial DNA cytochrome b gene and 5 nuclear microsatellite markers from 182 meat and skin samples of unidentified small cetaceans collected at three Peruvian markets between July 2006 and April 2007. Our results, compared to past surveys, indicate that *Lagenorhynchus obscurus*, *Phocoena spinipinnis*, *Tursiops truncatus*, *Delphinus capensis*, and *D. delphis* continue to be caught and marketed, but that the relative incidence of *P. spinipinnis* is highly reduced, possibly because of population depletion. The small number of possible sampling duplicates demonstrates that a high monitoring frequency is required for a thorough evaluation of incidental catches in the area. A wide public debate on by-catch mitigation measures is greatly warranted in Peru.

**Keywords** By-catch mortality · Cetacean · Microsatellites · Mitochondrial DNA

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## Introduction

Since the development some 60 years ago of cheaper and stronger fishing gear, fishing became much less selective (Clapham and Van Waerebeek 2007): a staggering amount of non-target marine life is hauled up every day with the catch, and then usually discarded overboard dead or dying. Occasionally, these animals, known as 'by-catch' or 'incidental catch' are subsequently and opportunistically

sold, but might ultimately become new targets of fishery activities. Over 300,000 small whales, dolphins, and porpoises die from entanglement in fishing gears each year, making by-catch the single largest cause of mortality for small cetaceans and pushing several species to the verge of extinction (Read et al. 2006). Drift and set gillnets pose the greatest threat to cetaceans due to their relatively low cost and ubiquitous use both in coastal and offshore waters (IWC 1994; Northridge 1996). Cetaceans become entangled when swimming into nets while transiting a body of water or while feeding too close to the fishing gear (Au WWL 1994). As many small whales, dolphins and porpoises are not strong enough to break free from most nets, entangled animals typically die from suffocation.

Monitoring and quantifying by-catches might help increase the pressure on involved parties to establish less destructive fishing practices. While attention has tended to focus on the control of legal and illegal international trade as a threat to biodiversity, much of the word's commerce in wildlife and fisheries is domestic and largely unregulated (Baker 2008). Small-scale fisheries, for example, employ over 80% of the world's 38 million fishers and provide about half of all fish caught for human consumption (Béné 2005). These fisheries occur primarily in developing nations, and their documentation and management are locally limited or non-existent, precluding evaluation of their impact on non-targeted fauna (Peckham et al. 2007). In such cases, surveys of large commercial markets, the end-point of the supply chain, can be an effective means of estimating true levels of exploitation or takes (Baker 2008). Maximizing the efficiency of such actions may involve the use of molecular genetic methods (when animals are not landed openly) for estimating the actual number of animals killed as well as for gathering basic parameters such as the species, the sex, and possibly the population of origin of each sampled individual (e.g., Cassens et al. 2005; Rosa et al. 2005).

By-catches and directed takes of small cetaceans in Peru have been monitored at regular intervals since 1985 (García-Godos 1992; Mangel et al. 2010; Van Waerebeek et al. 1997, 1999, 2002; Van Waerebeek and Reyes 1994a, b). Early studies clearly pointed to increasing numbers of captured animals; from some 10,000 in 1985 to approximately 14,000 in 1989 (Read et al. 1988; Van Waerebeek and Reyes 1994a, b) suggesting that regulatory measures should be taken. In 1990, a ministerial decree outlawed the exploitation of small cetaceans, but this rule was widely ignored and total annual catches reached an estimated 15,000–20,000 specimens in the period 1990–1993 (Van Waerebeek and Reyes 1994b). The Peruvian Ministry of Fisheries issued a stricter decree in 1994, that was accompanied by greater efforts to publicize and enforce the measures; as a result, overt landings ceased and cetacean meat gradually disappeared from view at public markets, although unknown quantities of meat were commercialized illegally. Finally, a 1996 national law (Anonymous 1996) prohibited entirely all captures and trade for five marine odontocetes (*Delphinus delphis*, *Delphinus capensis*, *Lagenorhynchus obscurus*, *Phocoena spinipinnis*, *Tursiops truncatus*) and two riverine dolphins (*Sotalia fluviatilis*, and *Inia geoffrensis*) (Van Waerebeek et al. 1997). Although these measures along with public campaigns, are likely to have undermined incentives for directed takes, they also seem to have pushed fishers to adopt new illegal practices (Mangel et al. 2010; Van Waerebeek et al. 2002). During the pre-ban era, entire carcasses of most animals taken (both accidentally and intentionally) were landed at the fishing ports to be butchered and sold, a practice that made it possible to record, quantify, study and sample these specimens (Read et al. 1988; Van Waerebeek and Reyes 1994a, b). Once the new protective regulations were enforced, by-caught specimens were either disposed at sea, butchered on board to bait longlines and gillnets that target large elasmobranchs (sharks and rays), or covertly landed as filleted meat for human consumption. It is thought that a minor proportion was still landed as carcasses at unsupervised sites along the coast. Simultaneously, the use of cetaceans as bait has been a rapidly expanding phenomenon given the high prices and low availability of traditional bait fish and the continuing supply of cetacean carcasses (Mangel et al. 2010; Van Waerebeek et al. 1997, 2002). Ironically, national legislation hardly influenced incidental captures, given that no adjustments in fishing techniques were established to mitigate net entanglements (Mangel et al. 2010; Van Waerebeek et al. 2002), but it made it far more difficult to record such events. Accordingly, the daily number of cetaceans that could be examined in local markets has drastically declined over the last 20 years (about 6 specimens per day in 1989, 2.5 specimens in 1999–2001, and less than one (0.6) in later years (Mangel et al. 2010; Van Waerebeek et al. 2002; Van Waerebeek and Reyes 1994a, b). On the other hand, an on-board survey in 2005–2007 showed that small cetaceans are still captured in high numbers, for instance with an estimated 2,600 animals per year taken just at the port of Salaverry (Mangel et al. 2010).

Here, we use molecular genetic approaches to analyse samples of meat and skin of unidentified small cetaceans collected at Peruvian markets in the fishing towns of Chimbote, Salaverry, and San José (Fig. 1), between July 2006 and April 2007. Chimbote has the greatest number of fish processing plants in operation in Peru (Berrios 1983). Salaverry is the port of Trujillo, the third largest city in Peru, and is the source of important small-scale fisheries on Peru's northern coast. The San José fishing community, on the other hand, is small and specializes in an inshore

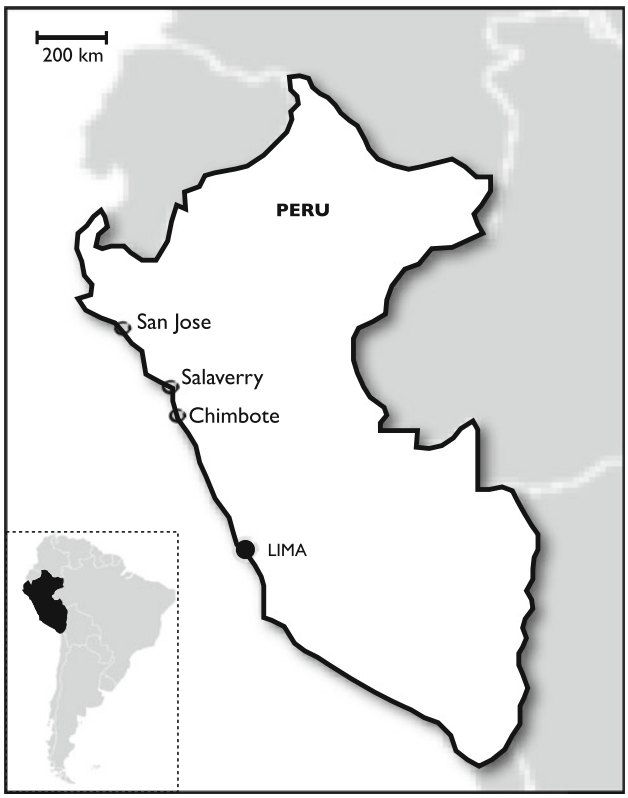


**Fig. 1** The three sampling locations in Peru (San Jose, Salaverry, Chimbote)

set-gillnet fishery, targeting several species of rays but resulting also in relatively high levels of by-catch of *P. spinipinnis* and other small cetaceans (Van Waerebeek and Reyes 1994b). We used molecular genetic analyses to determine (*i*) the relative species composition of the sampled animals, (*ii*) if individuals were sampled more than once, and (*iii*) the sex of each sampled animal. The results obtained were compared to those from an onboard observer program conducted on Salaverry-based boats during the same time-period (Mangel et al. 2010) and to past market surveys.

## Materials and methods

### Sampling

A set of 265 samples was collected at three different fish markets of northern Peru located in Chimbote, Salaverry, and San José, over a period of 10 months (July 2006–April 2007) (Fig. 1; Supplementary Table 3). Sampling was conducted on a daily basis in Chimbote and Salaverry markets from July to November 2006, subsequently only the Chimbote market was sampled. We only had access to pieces of cetacean meat derived from undetermined species except in the Chimbote market where, on rare occasions, complete animal carcasses were sampled. Local names of potential source species were collected from butchers. The sampling at the San José market was opportunistic and occurred once or twice a week during the study period. All tissue samples (mainly muscle) were conserved in sterile plastic bags filled with NaCl crystals for periods of 3–12 months. Samples were then washed overnight in PBS (pH 8), and stored in 100% ethanol. DNA was extracted using the DNeasy 96 Blood & Tissue Kit (QIAGEN). The quality of the extraction product was verified on a 1% agarose gel and quantified using NanoDrop (ThermoScientific). Although DNA yield and quality was highly variable among samples, they were all subjected to additional molecular analyses.

### Species identification

Since the first report on the existence of nuclear mitochondrial pseudogenes (numts) in the genome of *Mus musculus* (Du Buy and Riley 1967), the list of species exhibiting such nuclear copies has constantly increased and includes representatives from a wide range of eukaryotic groups (Bensasson et al. 2001). The presence of numts in cetaceans has not been confirmed yet, but is not unlikely; we thus decided to PCR amplify a large mitochondrial fragment to reduce the risk of co-amplifying nuclear copies (a method also employed by Xiong et al. (2009)): a ~3 kb fragment of the mitochondrial genome (including Cytochrome b, the Control Region and the adjacent tRNAs) was amplified by PCR, using primers '*tRNA Glu/L*' (5′-GTCTCACATGGACTYYAACCATGACCAATGA-3′) and '*12S/H*' (5′-GGGTTTATCGRTTAYAGAACAGGCTCCTCT-3′). Amplification products were verified on a 1% agarose gel before their purification using the QIAquick 96 PCR Purification Kit (QIAGEN). Cycle sequencing was performed on one strand using a shorter version of '*tRNA Glu/L*' primer ('*tRNA Glu/L_seq*' = 5′-GTCTCACATGGACT-3′). Sequencing products were run on an ABI 3730 48-capillaries sequencer. Sequence quality was verified using CodonCode Aligner (CodonCode Corporation).

As a first approximation, species identity was determined using the NCBI nucleotide BLAST algorithm, optimized for highly similar sequences ('*megablast*' option): the species of the query sequence was considered to be associated with the hit sequence with the lowest e-value. However, phylogeny-based analysis is a much more robust approach (than best BLAST hits) of species delineation (e.g., Milinkovitch et al. 2002). Using the NCBI BLAST interface, we checked the position of each query sequence in the "Fast Minimum Evolution" tree. However, this approach only allows seeing each sequence in isolation and the tree inference method is rather rudimentary. All sequences were then submitted to

the 'Witness for Whales' service of "DNA Surveillance", an online tool for species identification using DNA sequence data, and phylogenetic techniques (Ross et al. 2003). The query sequences were aligned using a simple profile alignment against the pre-aligned data set of reference sequences ("All cetaceans v.4.3" dataset), evolutionary distances among aligned sequences were calculated using the F84 model of nucleotide substitution, a neighbor-joining (NJ) tree was built, and a bootstrap analysis was performed using 1,000 pseudo-replicates to assess the robustness of the resulting nodes.

Furthermore, we performed Maximum Likelihood and Bayesian phylogenetic analyses (using MetaPIGA 2 v.18beta and MrBayes v.3.1.2, respectively) including unique haplotypes from all newly-sequenced specimens, as well as sequences from individuals of known species identity. Only delphinoid species were incorporated as reference sequences and the tree was rooted with the clade comprised of the Amazon river dolphin ('Boto'; *I. geoffrensis*) and 'Franciscana' (*Pontoporia blainvillei*) (Cassens et al. 2000). Maximum Likelihood analyses were performed with MetaPIGA2 (v.18beta) using 1,000 replicated metaGA searches (Lemmon and Milinkovitch 2002) with probability consensus pruning among four populations. The 4,000 resulting trees were used to compute a majority-rule consensus tree and calculate branch support values. Bayesian analyses were performed using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003) with four chains run simultaneously for $1.2 \times 10^6$ generations; trees were sampled every 100 cycles. Bayesian posterior probabilities were estimated as the majority-rule consensus tree among the 9,000 last sampled trees (3,000 samples discarded as burn-in). In both MetaPIGA and MrBayes, the general time reversible (GTR) nucleotide substitution model with gamma-distributed rate variation across sites and estimated proportion of invariable sites was applied separately to each of the three data partitions corresponding to the first, second, and third codon positions. Finally, we built intra-specific haplotype networks (using our sequences along with haplotypes already available at NCBI) with TCS v. 1.21 (Clement et al. 2000).

To discriminate *D. delphis* from *D. capensis* samples, we aligned the *Delphinus* Cytochrome b sequences available in NCBI (4 *D. capensis* and 46 *D. delphis* individuals) and identified 3 fixed mutations—positions 14 (G vs. A), 370 (A vs. G), and 520 (C vs. T)—for *D. delphis* versus *D. capensis* (positions numbered based on the sequences of Rosel et al. (1994)). The discriminating mutation at position 14 has already been described by Rosel et al. (1994) but not the other two. Note that, given the difficulties to discriminate the two species morphologically, we excluded from our analyses sequences for which species identification is not confirmed in a peer-reviewed publication (e.g., we have not incorporated sequences EF061405 and DQ320765, originally identified as '*D. tropicalis*'). On the basis of these 3 discriminating nucleotide positions, 78 of our Peruvian samples were identified as *D. capensis* and 11 as *D. delphis*. These results are consistent with our BLAST and phylogenetic analyses and, generally, with the documented large preponderance of *D. capensis* in Peruvian catches compared to *D. delphis* (Van Waerebeek 1994).

## Genotyping

Polymorphism at 18 microsatellite loci isolated from *P. spinipinnis* (9 loci) and *L. obscurus* (9 loci) was tested on a selection of 14 samples (representing the 5 species identified) and 2 control samples of known species (*P. spinipinnis* and *L. obscurus*), as described in Rosa et al. (2005) and Cassens et al. (2005). The 5 most polymorphic loci (PS1, PS2, PS7, PS8, and PS10, all isolated from *P. spinipinnis*) with successful cross-species amplification and distinct electrophoresis patterns were targeted for genotyping all our Peruvian samples.

Samples that: (*i*) were attributed to the same species, (*ii*) had the same sex, (*iii*) had the same multi-locus genotype, and (*iv*) were sampled at the same market, were considered as possible duplicates of the same individual. To validate further this result, the putative duplicates were genotyped for two additional *L. obscurus*-specific microsatellite loci (LDi24 and LDi47).

## Sexing

Individuals were sexed using a PCR assay targeting two loci simultaneously (Rosel 2003): a 382-bp fragment of the ZFX gene is expected to be amplified both in males and females, whereas a smaller fragment (339 bp) of the SRY gene should only be amplified in male individuals. The amplification pattern was verified by loading 10 µl of the PCR product on a 3% agarose gel. The efficiency of the PCR was first checked on previously collected samples of known gender (19 *L. obscurus*, 20 *P. spinipinnis*, and 5 *T. truncatus*) and the amplification was performed twice on 72 re-extracted individuals to verify the reproducibility of the results. Note that, as previously identified (Rosel 2003), the interpretation of these results was difficult because the two PCR products have similar sizes and the band intensities vary widely. Sex inference was easier for the control samples, most probably because of the high-quality genomic DNA available.

Given the previously mentioned difficulties and the highly skewed male:female ratio observed (cf. "Results" section), two additional PCR assays, partially validated for cetacean samples, were tested: (*i*) the co-amplification of a ZFX fragment (the same as above) and a ZFY fragment

(present only on the Y chromosome—269 bp), as described by Bérubé and Palsbøll (1996), and (*ii*) the co-amplification of an amelogenin exon (present on the pseudo-autosomal part of sex chromosomes—550 bp) and DBY7 (Y-linked intron—350 bp), as described in Hellborg and Ellegren (2003). One hundred and eleven samples were tested with the first approach and 75 with the second; in both cases, the results were identical with those obtained with the ZFX-SRY assay. Several additional pairs of primers targeting different portions of the SRY gene were designed and tested on the control samples, as well as on a minimum of 24 samples from this survey, always yielding the same sex-assignment results.

## Results and discussion

After DNA extraction, that yielded considerable variation in quality and quantity of genomic DNA, all 265 Peruvian samples were subjected to PCR targeting a 3 kb mtDNA fragment including Cytochrome b, the Control Region and adjacent tRNAs. Lack of amplification was observed only for four samples, even after a second DNA extraction was performed, probably due to the bad quality of the starting material. After direct sequencing of a ~700 bp fragment of the Cytochrome b gene and genotyping of 5 microsatellite loci, 74 samples were excluded from subsequent analyses because of the presence of numerous double-peaks in the sequencing electropherograms, and/or ambiguous genotypes. It is likely that double peaks originate from the co-amplification of nuclear mitochondrial pseudogenes (Tzika et al. in preparation).

### Species identification

Species assignment of sequences was first estimated using an approximate phylogenetic method implemented into NCBI BLAST (cf. "Materials and methods" section) and then validated with the 'Witness for Whales' online tool of the "DNA Surveillance" database (Ross et al. 2003). The two approaches yielded identical results listed in Table 1. Note that the species distribution per location varies; all *D. delphis*, and most of *D. capensis* and *P. spinipinnis* were sampled in Chimbote, whereas *L. obscurus* and *T. truncatus* samples were more evenly distributed between Chimbote and Salaverry. The relatively large proportion of *P. spinipinnis* sampled in San José is compatible with the fishing techniques traditionally used in that location (cf. "Introduction") as well as data from a 1993 port survey where cranial evidence indicated that 79% of small cetaceans taken (n = 71) were Burmeister's porpoises (Van Waerebeek and Reyes 1994b).

We also performed extensive Bayesian and metaGA phylogenetic analyses for species assignment of samples. Both analyses yielded the same topology as far as the localization of our haplotypes is concerned. The MetaPIGA cladogram shown in Fig. 2 confirms species assignment performed above as each sample groups with the reference sequence of the expected species. Visual species assignment by butchers or fish mongers (available for most samples) is in agreement with our molecular results in only 47% (85/182) of the cases. Among these matching specimens, 56 were *D. capensis* samples.

In addition, we compared our results with those from previous surveys in 1993, 1994, and 1995 (Table 1). Although the same species were generally identified, the relative species composition varied. Sampling in the San José market has always been restricted, but at the four recorded time points, the same species were observed. In accordance with Salaverry being considered the northern distribution limit of *L. obscurus* in the Southeast Pacific (Van Waerebeek et al. 1997), we identified no specimen of this species at San José whereas an increase in the relative abundance of the species in comparison with earlier periods was observed at Salaverry, compatible with the on-board survey conducted in parallel (Mangel et al. 2010). On the other hand, a decrease over time of the relative abundance was detected for *P. spinipinnis*, both at San José and Salaverry, but it was more dramatic at the latter location (from 74–79% in 1993–1995 to 3–6% in 2006–2001). The percentage of *Delphinus* spp. in Salaverry seems to be relatively constant over the years when the 'market' observation is taken into account, but a great fluctuation is implied by the 'on-board' study. The reverse pattern (high percentage of the species in the market and low percentage for the 'on-board' study) is observed for *T. truncatus*. Finally, when our results are compared to those of past surveys in the wider area of northern Peru (Supplementary Table 1), the following trends of relative incidence are observed: a decrease for *P. spinipinnis*, and an increase for *L. obscurus*. These fluctuations can be due to shifts in species distribution (Van Waerebeek et al. 1999), changes in preferred fishing grounds (inshore vs. offshore habitat, lower vs. higher latitudes), or to population depletion. Furthermore, relative species incidence varies between 'market' and 'on-board' campaigns but this could be due to greater sampling efforts at markets between July and November 2006. Indeed, relative catch compositions are strongly influenced by seasonal and opportunistic movements of cetacean populations and by the seasonal variation of harvesting techniques (e.g., artisanal fishing activities tend to shift from offshore in the summer to inshore grounds in the winter (Van Waerebeek and Reyes 1994a, b)).

**Table 1** Species assignment of samples per location and time period (number of individuals and percentage)

| | *D. capensis* | *D. delphis* | *L. obscurus* | *P. spinipinnis* | *T. truncatus* | Other | No. samples | Source |
|---|---|---|---|---|---|---|---|---|
| San José | | | | | | | | |
| 1993 | 11 (15.5%) | 0 | 0 | 56 (78.9%) | 2 (2.8%) | 2 (2.8%) | 71 | Van Waerebeek and Reyes (1994b) |
| 1994 | 2 (28.6%) | 0 | 0 | 5 (71.4%) | 0 | 0 | 7 | Van Waerebeek et al. (1997) |
| 1995 | 4 (33.3%) | | 0 | 8 (66.7%) | 0 | 0 | 12 | Van Waerebeek et al. (1999) |
| 2006–2007 (market) | 3 (42.9%) | 0 | 0 | 4 (57.1%) | 0 | 0 | 7 | Present study |
| Salaverry | | | | | | | | |
| 1993 | 2 (16.7%) | 0 | 0 | 9 (75%) | 0 | 1 (8.3%) | 12 | Van Waerebeek and Reyes (1994b) |
| 1994 | 9 (12.3%) | 0 | 2 (2.7%) | 54 (74%) | 7 (9.6%) | 1 (1.4%) | 73 | Van Waerebeek et al. (1997) |
| 1995 | 5 (14.7%) | | 1 (2.9%) | 27 (79.4%) | 0 | 0 | 34 | Van Waerebeek et al. (1999) |
| 2006–2007 (market) | 4 (12.9%) | 0 | 9 (29%) | 1 (3%) | 17 (54.8%) | 0 | 31 | Present study |
| 2005–2007 (on-board) | 120 (47.4%) | | 73 (28.9%) | 16 (6.3%) | 33 (13%) | 11 (4.4%) | 253 | Mangel et al. (2010) |
| Chimbote | | | | | | | | |
| 1993 | 132 (49.8%) | 0 | 0 | 119 (44.9%) | 13 (4.9%) | 1 (0.4%) | 265 | Van Waerebeek and Reyes (1994b) |
| 1994 | 20 (38.5%) | | 21 (40.4%) | 2 (3.8%) | 6 (11.5%) | 3 (5.8%) | 52 | Van Waerebeek et al. (1997) |
| 1995 | 0 | 0 | 0 | 10 (100%) | 0 | 0 | 10 | Van Waerebeek et al. (1999) |
| 2006–2007 (market) | 71 (49.3%) | 11 (7.6%) | 15 (10.4%) | 28 (19.4%) | 19 (13.2%) | 0 | 144 | Present study |

For the period 1993–1995, the assignment is morphology-based. Whenever it was not possible to differentiate *D. capensis* and *D. delphis*, the number of *Delphinus* spp. is indicated

## Haplotypic networks

We constructed, separately for each species, an haplotypic network including all our sequences and those available at NCBI. In our Peruvian samples, we identified six haplotypes for *D. capensis* with 90% (70/78) of the individuals represented by 2 haplotypes ('H1' and 'H2', Fig. 3a). Four published Cytochrome b *D. capensis* haplotypes are available at NCBI, two of which (U02674 and AF084087) correspond to 'H1' and the two others (U02675 and AF084086) share an haplotype (marked by an asterisk in Fig. 3a) not observed in our sample. Only one NCBI sequence (U02675) has a known geographical origin: a "Californian beach". As expected from the continuous distribution of *D. capensis* in coastal waters of the Humboldt Current (at least south to 28°S, Chile), no population differentiation among Peruvian sampling locations could be identified.

We identified only two haplotypes ('H1' and 'H2'; Fig. 3b) for the 11 *D. delphis* individuals in our sample. Forty-six Cytochrome b *D. delphis* sequences are available from previous studies, two of which (U02669 and U02670) overlap with the 'H1' haplotype identified in our survey. Both of these samples were collected in California. From the haplotypic network (Fig. 3b), there is evidence of gene flow, or retention of ancestral haplotypes, among the different *D. delphis* populations around the world. For example, specimens sharing the most abundant haplotype (Fig. 3b) originate from the North and South Pacific, the North Atlantic, and the Black Sea. These results are consistent with previous studies (Amaral et al. 2007; Natoli et al. 2006). Note the existence of the mysterious 'Group X', described by Amaral et al. (2007), formed by four females of the Eastern Atlantic, and separated from the other sequences by 7–9 mutations.

Seven haplotypes were identified for *L. obscurus*, although only 24 individuals were sampled. As for *D. capensis*, no clear population differentiation among Peruvian sampling locations could be evidenced. Four of the seven haplotypes correspond to the most common haplotypes already identified in an extensive population genetics study on dusky dolphins (Cassens et al. 2005); 'H1' (this study) corresponds to P5.1 and P5.2, 'H2' to P3.1, 'H4' to P2.1, and 'H6' to P1. On the other hand, 'H3', 'H5', and 'H7' represent sequences that had not been identified thus far. Note that the haplotypic network of Fig. 3c only comprises the Peruvian haplotypes, and it does not cover the whole species distribution. Indeed, as shown by Cassens et al. (2005), the Peruvian waters host a distinct population that does not share any haplotype with the remaining southern hemisphere populations, thus warranting special conservation and management status.

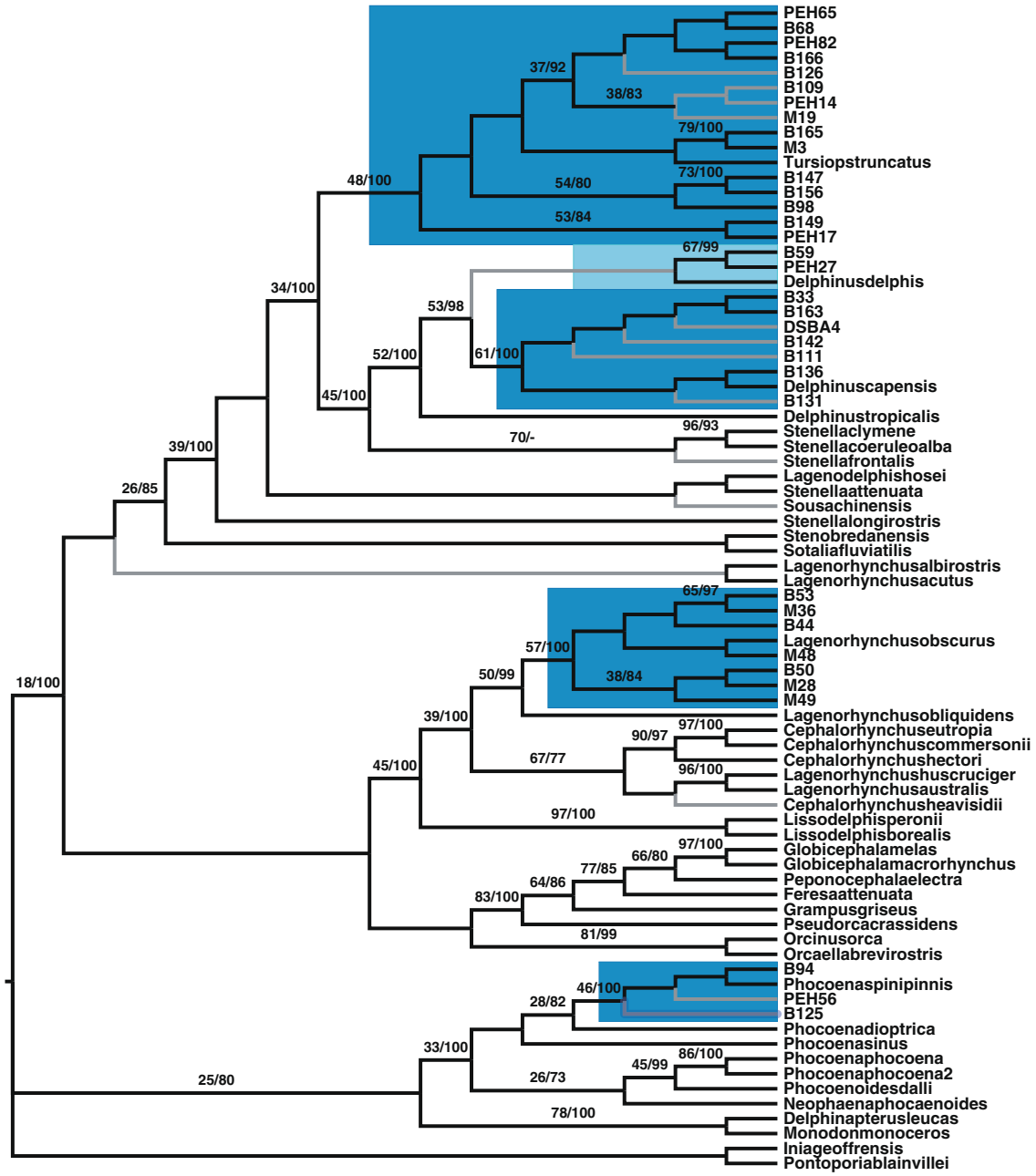


**Fig. 2** Cladogram among unique mtDNA cytochrome *b* haplotypes of the analysed Peruvian cetacean samples, along with delphinidae sequences available at NCBI. The MetaPIGA topology is shown. In *grey* are the branches that differ between the MetaPIGA and MrBayes consensus topologies. *Numbers on branches* indicate MetaPIGA support values and MrBayes posterior probabilities, when at least one of the two values is greater than 70%. Highlighted are the clades containing the haplotypes identified in this study. Sample IDs and sequence accession numbers are listed in the Supplementary Table 3

Only three haplotypes were identified for *P. spinipinnis* (with 30, 2 and 1 representatives, respectively; Fig. 3d) and no obvious population differentiation among sampling locations was evidenced. Two Cytochrome b Peruvian *P. spinipinnis* sequences are available at NCBI (U09676 and U13144), and correspond, respectively, to haplotypes 'H1' and 'H3'.

The greatest number of haplotypes (15) was identified from the analysis of the 36 *T. truncatus* samples (Fig. 3e). Fourteen Cytochrome b *T. truncatus* sequences are available at NCBI, and only two (U13145 and AF084094) correspond to an haplotype ('H7') of our samples. Clearly, *T. truncatus* exhibits the largest haplotypic diversity among the species investigated here. Moreover, the network exhibits: (*i*) a

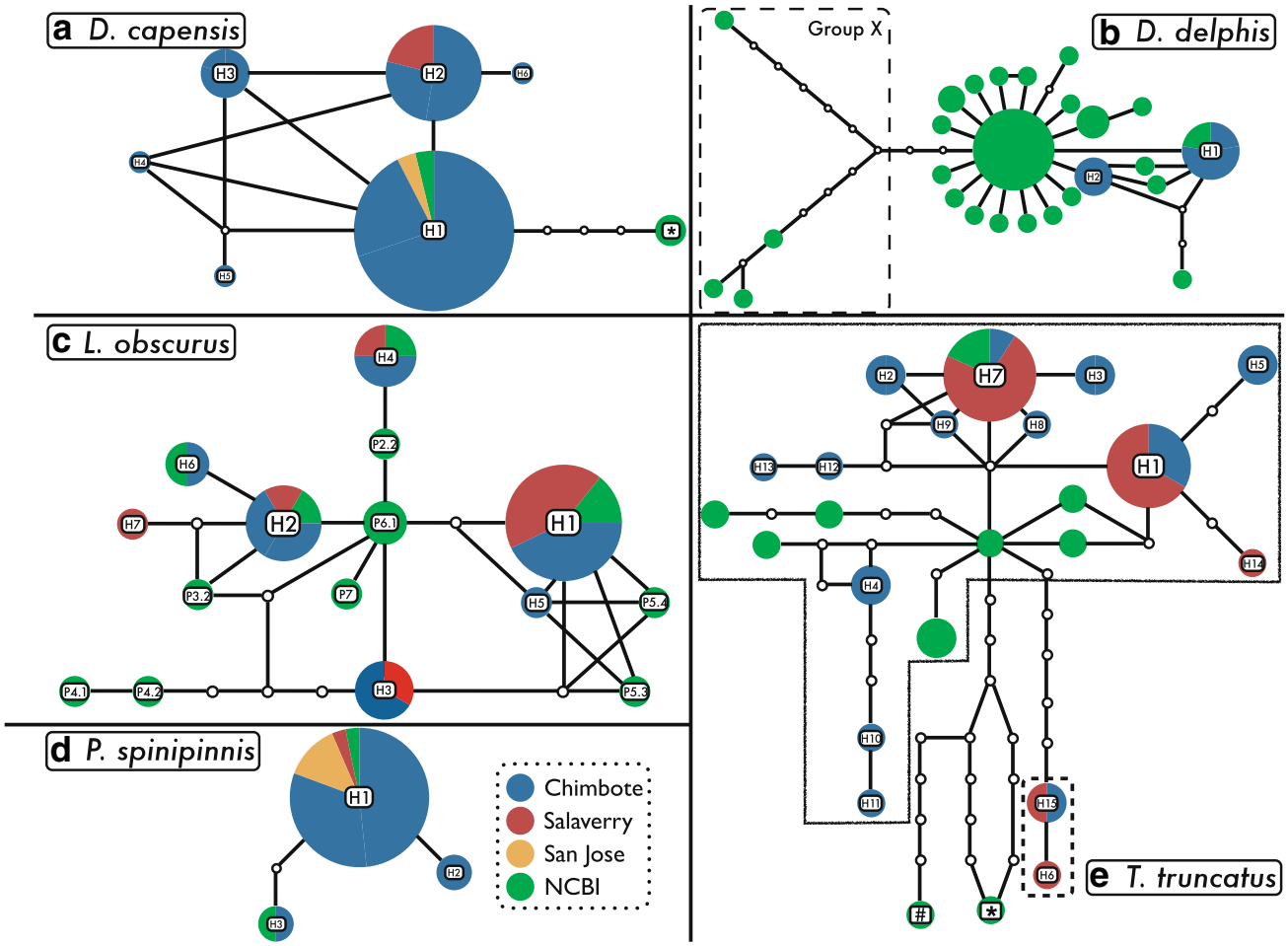


**Fig. 3** Species haplotypic networks among our Peruvian samples and those from conspecifics available at the NCBI public database. The surface of the *circles* is proportional to the number of individuals in which the corresponding haplotype was identified. *White circles* represent missing intermediate haplotypes. In the *D. capensis* network, the *asterisk* marks the haplotype of a sample collected in California (NCBI sequence U02675). In the *T. truncatus* network, the *asterisk* indicates a sample from the Gulf of Mexico whereas the hash sign indicates a sample of unknown origin

'central' group of haplotypes including samples from unknown locations as well as from regions as diverse as Peru, New Zealand, the Atlantic, and the Pacific Ocean, and (*ii*) two divergent lineages from Peru (dotted frame; Fig. 3e) and the Gulf of Mexico (asterisk; Fig. 3e). This *T. truncatus* network is consistent with morphological, ecological (feeding, parasites), behavioral, and distributional evidence (Van Waerebeek et al. 1990), and is supported by preliminary results of mtDNA control region sequencing (Sanino et al. 2005) indicating the existence of a wide-ranging and abundant offshore population off Peru and Chile, distinct from smaller inshore populations. Furthermore, globally, this result is also in accordance with the suggestion that the species includes intermingled offshore populations from all over the world, and distinct (genetically isolated) inshore clusters (Tezanos-Pinto et al. 2009). We suggest that the central group in our network represents offshore animals from multiple locations (many Peruvian specimens are likely taken by industrial purse-seiners), whereas the divergent lineage represented by 'H6' and 'H15' haplotypes may correspond to the Peruvian inshore population (Sanino et al. 2005; Van Waerebeek et al. 1990). The sample from Mexico (asterisk in Fig. 3e) probably belongs to a local 'inshore' population that has already been characterized both morphologically (Turner and Worthy 2003) and genetically (Tezanos-Pinto et al. 2009). The haplotype marked with a hash sign (Fig. 3e) is associated with the latter sample but is of unknown geographical origin.

## Sex bias

The ZFX/SRY PCR assay for sexing individuals worked for all sequenced samples, except 12. The sex ratio is highly skewed for all species (about 80:20% males:females). This result was further confirmed using the

ZFX/ZFY PCR essay on 111 samples and by the amelogenin/DBY7 PCR essay on 75 samples. It is therefore unlikely that the observed skewed sex ratio is due to amplification artifacts. However, and contrary to the species assignment assays, these results should be taken with great caution because pseudo-autosomal regions have not been fully characterized in cetaceans. Furthermore, several difficulties were encountered with the *P. spinipinnis* samples, where, for example, the amelogenin fragment could not be amplified.

Figure 4 shows that more males were sampled at most periods of the survey although this general trend is reversed at certain periods (October for *D. capensis*, December for *P. spinipinnis*, and November to February for *T. truncatus*). Given the small sampling size it is difficult to assess the significance of these variations. Table 2 summarizes the molecular sexing results per location and per species. The greatest percentage of males was sampled at Salaverry. Sex information (provided by fishers) was available for 30 of the 31 samples collected in Salaverry, and the males:females ratio was also found skewed with 63% (19/30) males. Molecular sexing was possible for 28 Salaverry samples, of which 27 had been morphologically sexed by fishers. In 19/27 cases, the morphological and molecular sex predictions were in agreement, and when these were not compatible (8 cases), a 'morphological' female switched to a 'molecular' male. However, the sexing by fishermen is not expected to be highly reliable.

These results are very different from the sex ratio determined by anatomical examination of complete carcasses by biologists during the 1989–1990 surveys in Pucusana, Peru, where an approximate identical percentage of males and females was found (based on 132 *L. obscurus*, 26 *P. spinipinnis*, and 6 *T. truncatus*). On the other hand, the results of the on-board observations in 2005–2007 also seem to suggest a higher percentage of males than females for all species except *T. truncatus*, although sample sizes are again too small for statistical significance assessment (Table 2). Note that recent analyses of samples from minke whales (*Balaenoptera acutorostrata*) harvested by the Japanese and Korean fleets also suggested a male-biased exploitation at certain areas (Baker et al. 2010). A similar result was obtained from a 17 years long survey on stranded bottlenose dolphins in the Mississippi sound region (Mattson et al. 2006), as well as for the short fin mako sharks in the southeast Pacific Ocean, resulting from yet unexplained sexual segregation mechanisms (Mucientes et al. 2009). The latter phenomenon has also been observed in several cetacean species (Brown et al. 1995; Laidre et al. 2009; Martin and da Silva 2004).

## Identification of multiple samples from single individuals

Based on 7 microsatellite loci (cf. "Materials and methods"), we identified 35 samples that could represent duplicates from a maximum of 16 individuals (Supplmentary Table 2). We can rule out duplicate set 15, because although samples 32 & 33, share the same genotype and sex, they are assigned to different species. Samples within each set of duplicates were obtained within a period of 1–32 days (Supplementary Table 2). Given that there are no means to conserve meat at the markets, it is highly unlikely that samples collected more than 4 days apart are real duplicates. Thus, only 8 individuals might have been sampled more than once (Supplmentary Table 2). This is very different from the situation in Korea where the half-life of whale meat is 1.8 months (Baker et al. 2007). This kind of information is critical, because it indicates that the optimal frequency with which markets should be monitored can greatly vary depending of the local storage practice, in addition to differences in muscle mass of the cetaceans involved (25–30 kg/dolphin compared to several tons/whale).

Our analysis indicates that the selected microsatellite loci isolated from *P. spinipinnis* and *L. obscurus* can be used for multiple Delphinoidea species. Two to 10 alleles per locus and per species were identified, and the average

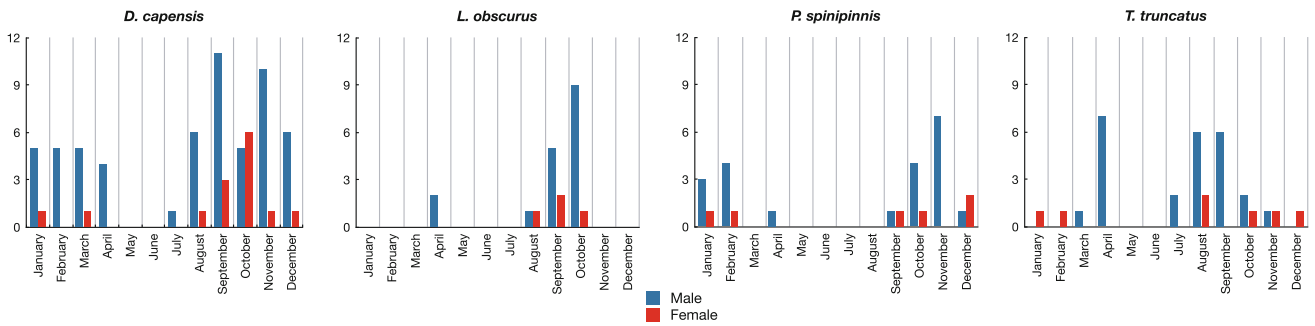


**Fig. 4** Number of males (*blue*) and females (*red*) sampled per month (combined for 2006 and 2007) and per species. The data is combined for the three Peruvian markets. Information on *D. delphis* is not shown due to small sample size (11 individuals)

**Table 2** Percentage of males (identified with molecular methods) per species and per market, compared to the percentage of males morphologically identified during the on-board surveys (only the animals by-caught during the time period of market sampling are considered)

| | San José | Salaverry | Chimbote | All markets (%) | On-board |
|---|---|---|---|---|---|
| *D. capensis* | 33.3% (1/3) | 100% (4/4) | 79.4% (54/68) | 78.7 | 60% (12/20) |
| *D. delphis* | – | – | 72.7% (8/11) | 72.7 | |
| *L. obscurus* | – | 85.7% (6/7) | 80% (12/15) | 81.8 | 59.1% (13/22) |
| *P. spinipinnis* | 75% (3/4) | 100% (1/1) | 76.9% (20/26) | 77.4 | 80% (4/5) |
| *T. truncatus* | – | 93.8% (15/16) | 62.5% (10/16) | 78.1 | 12.5% (1/8) |
| Total | 57% (4/7) | 93% (26/28) | 76.5% (104/136) | | |

number of alleles ranged from 3.8 (*D. delphis*) to 8.4 (*D. capensis*) (Table 3). Finally, private alleles (i.e., alleles found exclusively in a given species) were identified for all species except *D. delphis* and diagnostic alleles (i.e., private alleles present in a species with a frequency $> 10\%$) were identified for *L. obscurus*, *P. spinipinnis*, and *T. truncatus* (Table 3). Note that the use of diagnostic alleles for species identification provides a more stringent criterion than the use of private alleles because the latter can be erroneous due to genotyping errors.

## Conclusions

In Peru, as in many other countries, legislation banning all takes including by-catch in fishing activities has, ironically hidden conservation problems and made population management more challenging because monitoring becomes highly problematic when fishers continue catching cetaceans but utilize or dispose of carcasses clandestinely (Collins et al. 2002; Haelters and Camphuysen 2009; Mangel et al. 2010; Van Waerebeek et al. 1999, 2002). Thus, analysis of interview data or official reports may lead to the erroneous conclusion that by-catch is rare or has largely been phased-out, as has been claimed for Peru. It is likely that sample collection at local markets as well as on-board observations are the only means to obtain reliable estimates of the extent of the phenomenon. However, on-board observer programs are expensive and can be difficult to implement and maintain (D'Agrossa et al. 2000; Mangel et al. 2010; Northridge 1996). So far, outside North America, Western Europe, Australia, and New Zealand, there have been very few observer programs designed to monitor cetacean by-catch (Reeves et al. 2005). Such programs would involve high costs for developing countries. Nevertheless, the by-catch produced in these areas should not be ignored.

Here we presented results from molecular analyses of samples collected in three Peruvian markets and compared them to existing data from past surveys, as well as to an on-board monitoring program that was conducted in parallel. Our results indicate that penalizing small-cetacean indirect takes in Peru has not eliminated the problem, but rather concealed it. A similar effect from equivalent regulatory measures has been observed for other marine species, like sea turtles and seabirds (Alfaro Shigueto et al. 2007, 2008; Awkerman et al. 2006), suggesting that legislative or other regulatory protective measures often have limited efficiency for reducing the impact of fisheries on marine fauna.

For by-catch reduction to be sustainable and operational, wide involvement of fishers and efficient communication about management objectives and methodology are of paramount importance. For different fisheries, circumstances and regions, numerous ways of reducing by-catch have been suggested (Reeves et al. 2005), from modifications of fishing gear (Dawson 1991; Goodson et al. 1994), use of active acoustic alarms or 'pingers' (Cox et al. 2003; Kastelein et al. 2001; Leeney et al. 2007), seasonal closures of specific fishery activities (Trippel et al. 1996) to complete closure of fisheries (UN General Assembly 1991).

**Table 3** Number of alleles for each of the five microsatellite loci, mean number of alleles per species (*mean*), and allelic richness (*AR*) expected for 11 individuals (i.e., the sample size of *D. delphis*)

| Species | PS1 | PS2 | PS7 | PS8 | PS10 | Mean | AR | Private and diagnostic alleles | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *D. delphis* | 2 | 4 | 3 | 4 | 6 | 3.8 | 3.8 | – | – | – | – | – |
| *D. capensis* | 7 | 10 | 8 | 7 | 10 | 8.4 | 4.5 | PS7/102 | – | – | – | – |
| *L. obscurus* | 4 | 7 | 5 | 11 | 6 | 6.6 | 4.9 | PS2/96 | PS8/93* | PS8/105 | PS8/107 | – |
| *P. spinipinnis* | 8 | 7 | 4 | 5 | 4 | 5.6 | 4.3 | PS1/128 | PS2/113 | PS7/82* | – | – |
| *T. truncatus* | 10 | 7 | 6 | 9 | 8 | 8 | 6.3 | PS1/107 | PS1/109 | PS1/116 | PS8/83* | PS10/145 |

Private alleles (locus name/allele size) and diagnostic alleles (*asterisks*) are indicated in the last column

Unfortunately, the most effective methods have so far been detrimental to the local fishery industry (Cai et al. 2005). This not only affects the fishermen's livelihoods, it also exacerbates the divide and decreases trust between scientists and managers on one hand, and fishermen and local communities on the other (Lowry and Teilmann 1994). Furthermore, measures to reduce by-catches of one species can endanger others: e.g., switching from gillnets to longline fleets could reduce the impact on small cetaceans, but would probably put local turtle populations at risk (Peckham et al. 2007). In nations, such as Peru, where by-catch levels of small cetaceans may reach population-threatening levels, a wide debate among the public, the stakeholders, and the experts about by-catch mitigation measures is greatly desirable.

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