INTRASPECIFIC STRUCTURE OF THE NORTHERN RIGHT WHALE DOLPHIN (*LISSODELPHIS BOREALIS*): THE POWER OF AN ANALYSIS OF MOLECULAR VARIATION FOR DIFFERENTIATING GENETIC STOCKS

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ABSTRACT

The northern right whale dolphin (*Lissodelphis borealis*) has experienced very high levels of fishery-induced mortality in international high-seas, large-scale driftnet fisheries, from about 38°N to 46°N, and 171°E to 151°W. Assessing the impact of these mortalities is difficult, however, because of the possible existence of a coastal population off California and the Pacific Northwest that is separate from offshore populations. To obtain quantitative measures of reproductive isolation between putative populations, a portion of the control region of the mitochondrial DNA (mtDNA) genome was sequenced in 65 geographically dispersed individuals, then analyzed in a nested ANOVA format. No evidence of geographically concordant population structuring was apparent. In addition, a Mantel test, examining pairwise correspondence between geographic and genetic distances among samples, failed to detect any evidence of isolation by distance. Because negative data such as these are often used in management decisions, a power analysis was conducted to determine the probability that a survey of comparable size would yield statistically significant results under a hypothetical but likely level of divergence between "bona fide" stocks. The analysis yielded an estimate of the rate of making a type II or beta error of about 10%.

RESUMEN

El delfín liso *Lissodelphis borealis* ha sufrido niveles de mortalidad muy alta en alta mar (alrededor de 38°N a 46°N y 117°E a 151°W) inducidos por una pesquería a gran escala que usa redes de deriva. Es difícil evaluar el impacto de esta mortalidad debio a que posiblemente existen poblaciones costeras frente a California y el Pacífico Noroeste que están separadas de las poblaciones de mar adentro. Con el fin de cuantificar el aislamiento reproductivo entre las supuestas poblaciones se obtuvo la secuencia de una porción de la región central del genoma del ADN de la mitocondria (ADNmt) en 65 individuos procedentes de varias regiones; estos datos se analizaron usando un análisis de varianza anidado. No se encontró evidencia de una estructura de la población con concordancia geográfica. Aun más, la prueba de Mantel, que examina correspondencia aparejada entre distancias geográficas y genéticas entre las muestras, tampoco detectó evidencia de aislamiento asociado al distanciamiento espacial entre los individuos. Debido a que datos negativos como los mencionados antes se usan para hacer decisiones en le manejo de las pesquerías, hicimos un análisis de potencia estadística; determinamos la probabilidad de que un reconocimiento de tamaño similar produzca resultados estadísticamente significativos con niveles de divergencia hipotéticos pero plausibles entre stocks (suponiendo diferenciación, entre éstos). A partir del análisis, se estima que la frecuencia de producir errores del tipo II o beta es de alrededor 10%.

INTRODUCTION

Although the northern right whale dolphin (*Lissodelphis borealis*) is the most common cetacean species in the bycatch of the high-seas, large-scale driftnet fishery in the North Pacific (Hobbs and Jones 1993), its intraspecific (stock) structure is unknown. A separate coastal population off California and the Pacific Northwest may exist, but there are insufficient data to allow a rigorous test of this hypothesis even though the species has been the focus of considerable biological effort and expense.

Yet it is axiomatic that knowledge of the intraspecific (stock) structure is a critical component in determining the status and subsequent management of species impacted by human activities; stock, not species, is the unit on which management parameters such as optimal sustainable population and allowable biological removal estimates are based. Both the Marine Mammal Protection Act and the Endangered Species Act direct management efforts at taxa below the species level. The concern of the responsible manager is that if stock structure is arbitrarily subdivided too finely, population estimates and allowable removal levels may be so small that local fisheries may be unnecessarily closed down. On the other hand, if a species is too coarsely subdivided, evolutionarily significant local populations may be depleted or destroyed.

Knowledge of stock structure comes from four classes of information: distribution, demography, morphology, and genetics (Waples 1991a, b; Dizon et al. 1992). What
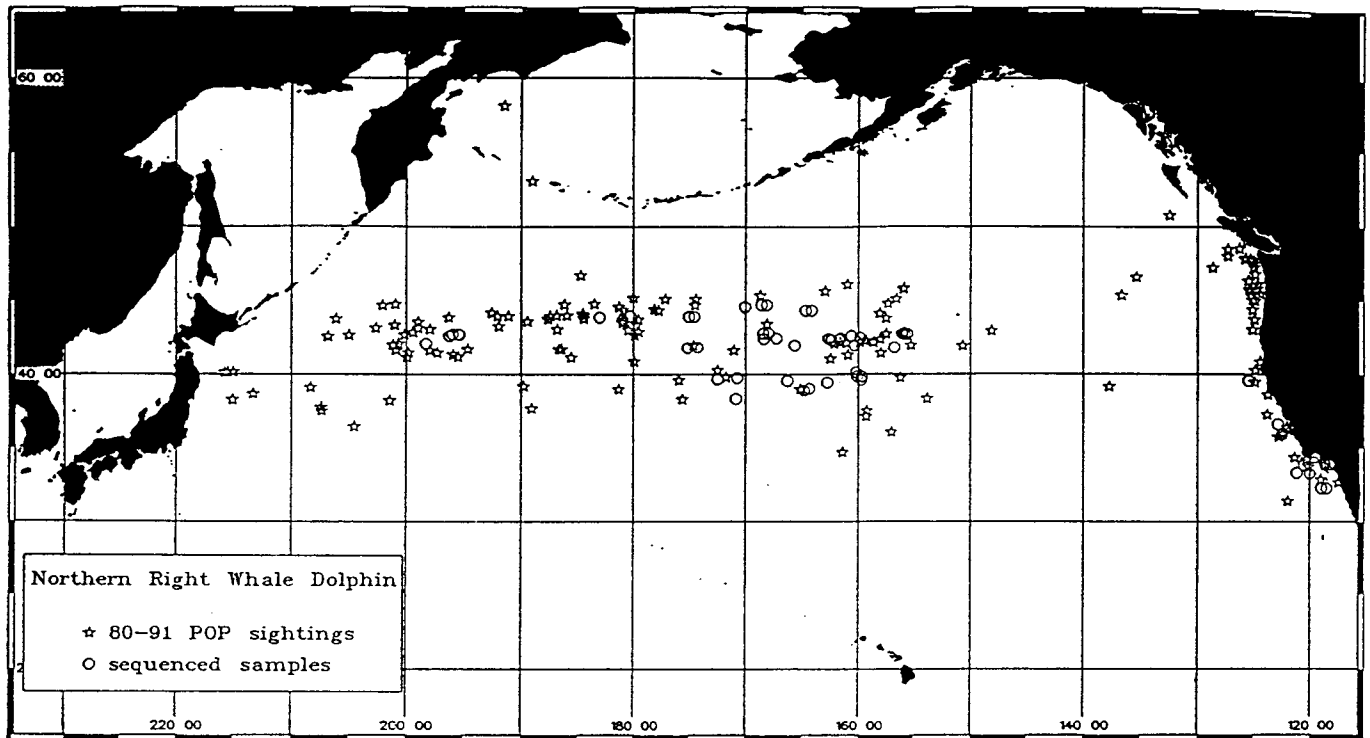
Figure 1. High seas sightings (*stars*) of northern right whale dolphins (*Lissodelphis borealis*). Data from the National Marine Mammal Laboratory's Platform of Opportunity Program (POP), 1980–91. *Circles* are sequenced sample collection locations.

few data are available from these sources for *L. borealis* are summarized in Leatherwood and Walker (1979), Baird and Stacey (1991), and Ferrero et al. (1993). Save for some distributional data that indicate an area of reduced density separating the potential coastal population and the offshore one (figure 1), there is no information in the literature regarding whether *L. borealis* exists as a single, panmictic population or is subdivided into two or more breeding populations. Although the tendency of delphinids to subdivide into coastal and offshore populations is well known (see Perrin et al. 1985; Dizon et al., in press), the stock structure of this species is an open question.

About 6,700 driftnet operations were observed between 1989 and 1990 (Hobbs and Jones 1993), and biological samples from the cetacean bycatch were collected. These, as well as samples collected off the U.S. west coast from beach-cast animals, cross-bow biopsies of bow-riding animals, and fishery mortalities were used in a molecular analysis of mtDNA.

Analyses based on molecular information derive their power from two properties (Vigilant et al. 1991): (1) the evolution of the molecule is dominated by selectively neutral substitutions, and (2) the changes occur at a reasonably constant rate, at least within fairly closely related taxa. The mtDNA molecule has the features of neutral and rate-constant mutation (Moritz et al. 1987). In addition, it is haploid and maternally inherited, eliminating

recombination effects (Giles et al. 1980). These properties greatly simplify data analysis, and the relatively high rate of evolution (Moritz et al. 1987; Hoelzel et al. 1991) allows resolution of closely related forms. Analyses of mtDNA, primarily restriction fragment length polymorphism analyses, but also including direct sequencing, have been extensively used in the last several years to study intraspecific structure of marine mammals (e.g., see Baker et al. 1985, 1990, 1993; Lint et al. 1990; Dizon et al. 1991; Rosel 1992; Dowling and Brown 1993; Hoelzel et al. 1993).

Here we report on the results of a study examining the genetic diversity within and among three putative populations of *L. borealis* sampled from locations spanning their known range. We conduct a power analysis to estimate the probability of making a type II or beta error—i.e., incorrectly accepting the null hypothesis that the population is not subdivided into stocks. From a conservation standpoint, the costs of making such an error are perhaps higher than the costs of making a type I error—incorrectly rejecting the null hypothesis—because of the potential for destroying an undetected but evolutionarily significant population component of the species. Using genetic diversity studies from other species, we attempt to estimate the minimum genetic differences that separate closely related but "bona fide" stocks, and we use that estimate to determine the statistical power of our current study.

MATERIALS AND METHODS

Fifty-two individuals from the bycatch of the high-seas driftnet fisheries were obtained by National Marine Fisheries Service (NMFS) observers from the National Marine Mammal Laboratory (NMML). Of these, 16 were from west of the dateline (figure 1). The majority of samples obtained from NMML were clustered to the east of the dateline but west of 155°W latitude; of those, we sequenced 36. Note that the classification of animals east and west of the dateline into separate populations is arbitrary and not supported by any significant hiatus in distribution. We also obtained 13 samples from coastal U.S. waters. These samples came from incidental kills collected by fishery observers from Southwest Region (SWR, NMFS) accompanying set- or drift-net boats, dead beach-cast animals collected under the auspices of the California Marine Mammal Stranding Network organized by the SWR, or projectile biopsies of bow-riding dolphins collected under NMFS permit from a research vessel.

Our specific DNA procedures were as follows (see also Rosel 1992): tissue samples were preserved and stored in a saturated NaCl solution of 20% DMSO at room temperature (Amos and Hoelzel 1991). We extracted total cellular DNA (nuclear plus mtDNA) using established protocols (Hillis and Moritz 1990) modified for our particular needs. Double-stranded (symmetric) PCR amplification of approximately 0.1 μg template (sample) DNA was carried out in 100 μl reactions by means of standard reaction conditions and reagents supplied with the GeneAMP kit (Perkin Elmer Cetus, Inc). Chain elongation was initiated with the addition of 30 pmoles of primers complementary to regions up- and downstream of the region of interest, the 5' end of the control region of the mtDNA genome—about a half of the control region. The upstream primer (anneals to heavy strand) was biotinylated at its 5' end to facilitate immobilization of the amplicon for later procedures. This primer is the so-called "universal primer" (slightly modified from Kocher et al. [1989] by Rosel [1992]). The downstream primer (anneals to light-strand), designed also by Rosel, anneals near similarity block B, a conserved region within the control region (Southern et al. 1988).

We used streptavidin-coated magnetic beads (Dynal Inc.; Hultman et al. 1989) to clean PCR-amplified samples and make them single stranded. The extremely high affinity of the streptavidin for the biotin moiety on the 5' end of the heavy-strand primer allowed the immobilization of the double-strand amplicon. A neodynmium-iron-boron permanent magnet was used to sediment the beads against a side of a 1.5 μl microcentrifuge tube for washing to remove unincorporated reaction products and for removal of the heavy strand itself by denaturization and subsequent elution.

We performed sequencing reactions on the light-strand DNA only, using standard conditions and reagents as supplied with the Sequenase, Version 2.0 sequencing kit (United States Biochemical Corporation) and 5 μCi of [α-^{35}S]dATP. Two internal primers, producing overlapping sequences, allowed us to routinely sequence about 500 bp's, about 80% of the amplicon. The sequencing reactants were then electrophoresed on 6% polyacrylamide wedge gels (0.4 to 0.8 mm) from 2 to 6 hours and read from autoradiographs exposed to the vacuum-dried gels. The individual sequences were aligned by eye in a spreadsheet format. Sequences will be submitted to GenBank.

Sequences were analyzed by means of distance methods, as opposed to parsimony methods. For our studies, parsimony methods were usually inappropriate for analyses of intraspecific structure. The number of taxa (individuals) far exceeded the number of phylogenetically informative sites (Stewart 1993), and a very large number of equally parsimonious trees were found, indicating the underlying lack of resolution of parsimony approaches for revealing intraspecific structure (Excoffier and Smouse 1994).

The genetic or evolutionary distance separating each pair of sequences was estimated using the Tamura-Nei method as implemented in Kumar et al.'s (1993) computer package, MEGA. The authors recommend this method as especially appropriate for mtDNA sequences. The derived matrix of 2,080 ($N[N-1]/2$ where $N = 65$) pairwise genetic distances was examined for evidence of a geographic structure, i.e., concordance between genetic distance and geographic strata, by means of an analysis of variance method modified for use with molecular sequence data (Excoffier et al. 1992). We used the computer program described by Excoffier et al. (AMOVA, analysis of molecular variance) and furnished by the senior author. The linear model employed assumes that individuals are arranged into populations, and populations nested into groups, defined by nongenetic criteria (sampling location). The program computes estimates of variance components and F-statistic analogues, designated Φ-statistics. The Φ-statistic is the correlation of random haplotypes drawn from within a stratum to random haplotypes drawn from among all the strata (Excoffier et al. 1992). The method also employs resampling methods to test the significance of the variance components and statistics, thus avoiding assumptions of normality. The method does not require specific assumptions regarding the evolution of sequences and, while not as powerful at detecting population subdivision as more rigorous models (e.g., Takahata and Palumbi 1985), is certainly more robust.

To ascertain if there was evidence of isolation by distance, we employed a Mantel test for matrix corre-
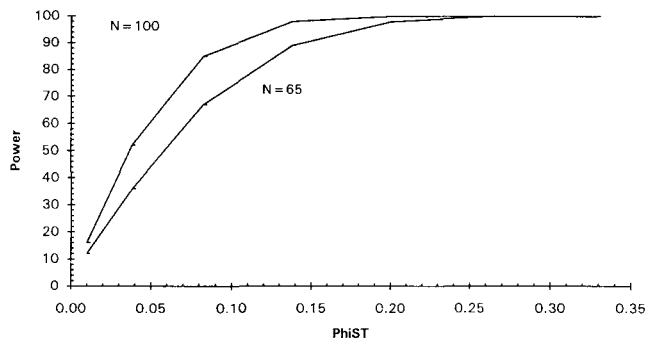
Figure 2. Power of an F (Φ) test at $\alpha = 0.05$ and 1 d.f. for a sample size of 100 and 65 (Cohen 1988). PhiST = Φ_{ST}.

spondence as implemented in the NT-SYS package (Rohlf 1990). A matrix of pairwise genetic distances was compared with a matrix of pairwise geographic distances. The geographic distances between samples were calculated as great circle distances in nautical miles. This program plots one matrix against the other in an element-by-element fashion and computes the Mantel statistic, Z, to measure the degree of relationship.

$$Z = \sum_{i,j}^{n} x_{ij} y_{ij},$$

where X_{ij} and Y_{ij} are the off-diagonal elements of matrices X and Y. If the matrices show similar relationships, Z will be larger than would be expected by chance alone. The Mantel test as implemented also uses resampling methods to test the significance of the variance components and statistics.

The variance estimates and the Φ-statistics from the AMOVA were used in a subsequent power analysis (Cohen 1988). We assume an alpha level of 0.05 with one degree of freedom and use the "Power of F" test tables in Cohen (1988) to construct a graph of the relationship between Φ_{ST} and power for two sample sizes ($N = 65$, $N = 100$; figure 2).

RESULTS

A total of 400 base pairs were sequenced in the control region plus the entire proline and a small portion of the threonine tRNA genes. The latter regions were eliminated from subsequent analysis. Of the 65 animals sequenced, one group of 5 and one group of 3 had identical haplotypes, and both groups contained animals from the coastal and the offshore populations. Seven other pairs each shared a unique sequence. For three pairs of the seven, both animals were sampled from the same school, so there is a potential that there were first- or second-order relations, but in the other four pairs, each member came from widely separate locations.

Of the 400 base pairs sequenced, there were 73 variable sites and 3 variable insertion/deletion events. Sixty-

TABLE 1
ANOVA on Pairwise Genetic Distances between *Lissodelphis Borealis* mtDNA Sequences Stratified into Three Putative Populations: Inshore, Offshore East of the Dateline, and Offshore West of the Dateline

| Component | Observed partition | | p | Φ-statistic |
	Variance	% total		
Among	0.000695	0.66	0.14	$\Phi_{ST} = 0.007$
Within	0.010388	99.34		

eight of these sites contained only transitional changes; three sites showed transversional changes, and two sites exhibited both. Average pair-wise Tamura-Nei genetic distances for the total sample were 0.020, or 2% ($N = 65$); for the inshore sample, 0.020 ($N = 13$); for the offshore sample east of the dateline, 0.021 ($N = 36$); and for the offshore sample west of the dateline, 0.022 ($N = 16$).

To analyze the effect of the relationship of geographic sampling location and genetic variation, we stratified the sequenced sample into three putative geographic populations: inshore, offshore west of the dateline, and offshore east of the dateline. The genetic distance measurements were then analyzed with the AMOVA program (table 1). The value p is the probability of having a more extreme variance component and Φ-statistic than the observed values by chance alone. The significance of the variance component and Φ-statistic is tested by permuting individuals to random populations. The within-population effect is tested by permuting individuals across populations without regard to either their original population or region.

It is apparent from table 1 that the main effect is due simply to variability among individual pairwise measurements and that there is no reduction in variance from stratification into populations. No other geographic stratifications of the data, including simple offshore and onshore populations, nor elimination of males from the analysis resulted in any significant effects. These results are consistent with lack of geographic structuring of the population.

Because of the disparity in size of geographic areas of the sampled populations (the offshore region spans in its east-west axis 1,862 n.mi., whereas the California region spans only 480 n.mi.), the AMOVA analysis may mask evidence of isolation by distance. Many offshore individuals are separated by larger distances than California samples are from some of the far eastern offshore samples. To examine this masking, we used the Mantel test (Rohlf 1990) to compare the same pairwise matrix of genetic distances used in the AMOVA analysis to a matrix of pairwise geographic distances. No correlation existed between genetic and geographic distance (normalized Mantel statistic $Z = -0.005$). Of 1000 permuted matrices, 49% demonstrated a larger Z value than the sample matrices, 51% a smaller value.

DISCUSSION

The degree of nucleotide variation within *Lissodelphis borealis* is somewhat higher than in other populations examined in our laboratory. In the portion of the control region sequenced, we found 76 variable sites. In the same region, Rosel (1992) found 43 variable sites in her sample of 29 *Delphinus delphis*, and those included individuals from two different ocean basins and two different morphotypes (long-beaked and short-beaked) that display fixed genetic differences and may be separate species. In her study, she estimated a genetic diversity of 1.2% for long-beaked samples and 1.6% for short-beaked samples.

For *Lissodelphis borealis*, the within-population diversity for the total sample was about 2.1% and not significantly different when calculated for either the offshore or California population alone. Neutral theory predicts a positive correlation between genetic diversity and population size (although this relationship can be profoundly altered by historical demography; Avise et al. 1988). The similarity in genetic diversity in the offshore and inshore samples is consistent with the hypothesis that the two samples were drawn from the same population or, alternatively, from populations of the same size. However, population abundance in the offshore region has been estimated to be about an order of magnitude larger than in the inshore region (Buckland et al. 1993; Barlow, in press; Barlow and Forney, in press).

Sequence divergence between *L. borealis* and other dolphin species seems reasonable when compared to values for closely related species pairs obtained in other similar studies. Sequence divergence between our study species and *Delphinus delphis* (Rosel 1992) is 6.7% and between our study species and *Stenella attenuata*, 7.5% (our data). Rosel (1992) estimated sequence divergence between *S. attenuata* and *D. delphis* of 6.0%.

Although the results of both the AMOVA and the Mantel tests, as well as the similarity of the genetic diversity values between the putative populations, are consistent with a lack of intraspecific structuring in *L. borealis*, it is negative evidence: Can a conservation manager develop policy from it? In other words, are these negative results of any value in deciding if the species should be managed as a single panmictic population or separately as an inshore and an offshore population? This is an important question because the putative offshore population was subject to high levels of fishing mortality and may well be depleted (Hobbs and Jones 1993), and the inshore population is currently exploited, albeit at a very low level (Forney, in press).

Mindful of the conservation dangers of incorrectly accepting the null hypothesis that the population is not subdivided into stocks, we attempted to estimate the power of our analysis to yield statistically significant re-

sults if the H_0 was indeed false. For this, we assume that given taxa levels, at least among closely related forms, are separated by roughly similar relative genetic distances; the higher the taxa level, the greater the genetic distance. If genetic distances are calculated from equivalent data sets, studies of genetic divergence between known stocks may be, at least as a first approximation, a way to "calibrate" stock differentiation, i.e., to estimate "effect size" (Cohen 1988).

We did this by determining the Φ_{ST} values obtained when comparisons are made of "bona fide" stocks examined experimentally in the same way as in our present study. However, here the reasoning becomes somewhat circular in that bona fide stocks are defined at least in part when they show statistically significant ($\alpha = 0.05$) Φ_{ST} values, but the biology and distribution of the two following stocks used to calibrate effect size make them likely calibration candidates. We use the Black Sea short-beaked common dolphins and eastern tropical Pacific (ETP) short-beaked common dolphins (Rosel 1992), $\Phi_{ST} = 0.15$, and the exclusively Washington–central California clade of northwestern Pacific harbor porpoise, which can be geographically separated into a northern and southern stock, $\Phi_{ST} = 0.18$ (Rosel 1992, and additional unpublished data from our laboratory). The harbor porpoise stocks are more differentiated than the common dolphin ones, although the common dolphin pair is clearly isolated by its extreme geographic separation. The harbor porpoise pair are less obviously separate stocks, but harbor porpoise are exclusively a coastal species, and recent contaminant analyses indicate little exchange between the two geographic regions (Calambokidis and Barlow 1991).

The question remains, however, is $\Phi_{ST} = 0.15$ a reasonable level to distinguish between biologically significant subdivisions and merely statistically significant ones? Although 0.15 is certainly arguable, it is not unreasonable to assume that some cutoff value for Φ_{ST} exists. The alternative is that any statistically significant value for Φ_{ST}, no matter how small, is evidence for restrictive gene flow, and any evidence of restrictive gene flow would be reason to subdivide a population for management purposes. This alternative would be a difficult one upon which to base management because no natural populations are likely to exhibit true panmixia. This would also mean that all genetic tests of stock have inherently low power because if the effect size for Φ_{ST} approaches zero, then the required sample size approaches infinity.

Rejecting the alternative, we for the moment consider the minimum value expected for Φ_{ST} for similar mtDNA analyses among similar taxa to be about 0.15. Then the statistical power of our present study ranges from 0.90 to 0.95 (figure 2). Put another way, the rate

of failing to reject a false null hypothesis of panmixia is 10%.

However, making a decision regarding stock structure of a species is a subjective one requiring considerable experience in order to weigh data from the four broad classes of information to infer the existence of evolutionarily significant populations, characterized by unique, adaptable but unmeasurable, genetic variation. In this study, genetic information from a neutral gene was sought as a proxy for evidence of adaptable population differentiation, and, like other biological information (Leatherwood and Walker 1979; Baird and Stacey 1991; and Ferrero et al. 1993), yielded no indication of population subdivision. And at the same time, we note that the power of our study was reasonably high.

But the validity of the interspecific calibration of stock differentiation, as well as the relatively small size of the coastal sample relative to the offshore sample size, warrants caution in accepting the null hypothesis of a single, panmictic stock for the northern right whale dolphin on the basis of the relatively high estimated power. In addition, an international moratorium on high-seas, large-scale driftnet fishery has reduced the urgency for such information. Perhaps the most important point of the study is the importance of considering power in stock designation studies. The nature of conservation management, with its need to balance conservation and economic interests, gives increased weight to negative results, which are usually dismissed in more academic investigations.

ACKNOWLEDGMENTS

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