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Genetic Difference in the Cytochrome *c* Oxidase I Gene Between "Typical" and Equatorial Forms of *Sthenoteuthis oualaniensis*

Abstract

This study aimed to make the first genetic comparison of the two major forms of the ommastrephid squid, *Sthenoteuthis oualaniensis*. These two forms are the widely dispersed, photophore bearing, "typical" form and the smaller, photophoreless, equatorial form. Samples were taken at seven stations between the latitudes of 20° N and 3° N in the central Pacific. Through correlating morphological data with RFLP and nested PCR analysis of the Cytochrome Oxidase I gene in *S. oualaniensis* this study has found strong preliminary evidence of a significant genetic difference between the two morphological varieties. This data may point to a species-level of phylogenetic divergence for the two forms currently classified as *Sthenoteuthis oualaniensis* .

Introduction

This study aimed to assay the genetic diversity of the *Sthenoteuthis oualaniensis* squid between Hawaii and the Line Islands, with the goal of determining what, if any, genetic population structure is exhibited by the species over this geographic range.

Despite significant study, a firm grasp on the population structure of *S. oualaniensis* remains elusive. Like other species in the Ommastrephid family, *S. oualaniensis* exhibits a relatively complex intraspecies population structure (Nesis, 1993 after Nesis, 1977). In fact, it has been suggested that *S. oualaniensis* exhibits the most complicated population structure currently known (Nesis, 1993). This derives largely from the fact that there are three to five morphological forms of varying distinctness that

exist across the species' range. These forms vary in their size, in the presence or absence of a light producing organ on the dorsal side of the squid, termed a photophore, and in the number of stiffening ribs, known as axes, on the mantle of the squid.

Under the most highly divided scheme the five forms are as follows: (1) a smaller, "dwarf" form that is unique in lacking the dorsal photophore, which inhabits equatorial waters; (2) a "giant" form, reaching sizes of 65 cm, which occupies the northern Arabian Seas and the Gulf of Aden; (3) a small form with the dorsal photophore, which occupies the Red Sea, Mozambique Channel and the Eastern Tropical Pacific; (4) a middle-sized form with double axes, which is found throughout the species range; and (5) a middle-sized form with single axes found in the Red Sea, Gulf of Aden, and the northern Arabian Sea (Young, 1998) (Figure 1). Under a more heavily lumped theory only three distinct forms are recognized: the dwarf photophoreless form, the typical form with both single and double axes, and the giant form (Nesis, 1993; Yatsu, 1998). Along the cruise track we sampled two of the five types: the equatorial form (1) and the typical form with double axes (4).

The taxonomic status of these three to five forms is complicated by largely unknown life cycles, spawning routines, and foraging habits and remains debated. At least three opinions exist as to the relation of these forms. One hypothesis states that these forms represent intrapopulational dimorphisms owing to the presence of early and late maturing forms. The second hypothesis suggests that these types belong to different populations and their morphological differences come from genetically controlled differences in maturation size. The third hypothesis suggests that the forms with and

without a dorsal photophore represent highly divergent, genetically distinct species (Nesis, 1993 after Nesis, 1985; Nigmatullin, 1983; Zuev, 1985).

A more recent hypothesis describes the population structure of *S. oualaniensis* as a nested, 3-layered, hierarchical regime with all forms of *S. oualaniensis* on the first layer, all photophore-bearing forms on the second layer, and a variety of geographically diverse forms with single and double axes on the third layer. This author also suggests that the dwarf photophoreless form may represent a nascent diverging species (Nesis, 1993).

Previous to this study little genetic data existed for this species and the lack of knowledge was especially high in the central and equatorial specific. Less than forty, unpublished DNA sequences exist for *S. oualaniensis* in the area around Hawaii and there is a sequence for only one individual along in the area between Hawaii and the Line Islands (Gilly, W.F., personal communication), which represents a large section of the squid's range. Interestingly, this lone specimen was found to possess a cyctochrome oxidase I (COI) gene that differed by approximately 8% from all other known sequences for this species (Gilly, W.F., personal communication). This genetically aberrant sample opens up the possibility for significant genetic diversity in the region.

Through collecting morphological data in conjunction with genetic data, this study has established a relationship between the genetic phylogeny and the morphology of the two sampled forms, suggesting that the "typical" and the photophoreless types are genetically divergent.

Materials and Methods

Adult samples of *S. oualaniensis* (1,2,4-8, 15-18) were collected with a line and jig setup at four sites and larval samples (9-14) were collected with a neuston net and meter net at three additional sites between the Hawaiian Islands and the Line Islands. One additional sample (3) was opportunistically collected as dropped prey from a booby. Squid were sacrificed by suffocation and data on their mantle length, mantle width, presence/absence of a dorsal photophore, the presence of single or double ventral axes, and their sex was recorded. A small tissue sample was removed from one of the arms of each squid and placed in 70% EtOH. All adult squid, except for samples 1 and 2 were preserved in a solution of 10% formalin buffered with deionized water.

DNA was then extracted from all tissue samples utilizing a Nucleospin kit. The extraction was performed according to the NucleoSpin Tissue Protocol except for the following differences: All reagents were used at half of the specified volumes, the first incubation was at 65 ° C at 2 hours, and all centrifuge spins were be done at 2000 Gs, except for the final spin, which will performed at 500 Gs. After the extraction was complete the samples were run out on a 1.5% agarose gel to confirm the presence of total DNA. A 1 kilobase ladder was used in this gel prep and in all other gels throught the study.

Restriction Fragment Length Polymorphism (RFLP) analysis was performed on samples 1, 2, 4-13. A PCR mixture was first created which contained, for each sample, 2.5 µl 10x buffer, 2.5 µl dNTPS at 200 µM, 1.5 µl LCO 1490 [3'-GGTCAACAAATCATAAAGAT ATTGG-5'], 1.5 µl HCO 2198 [5'-TAAACTTCAGGGTGACCAAAAAATCA-3'], 0.06 µl AMPLItaq polymerase, 1 µl

sample DNA, and PCR grade H_2O added to obtain a 25 μl reacting volume. Samples were then covered with mineral oil to prevent evaporation and were run on a MJ PTC-150 PCR machine for 94˚C (2 min), followed by 34 cycles of 94˚C (30s), 50˚C (30s), and 72˚C (1 min) followed by a final extension at 72˚C (7 min). PCR products were then run-out on a 2% agarose gel impregnated with EtBr to ascertain the presence or absence of PCR product.

Two restriction enzymes, BsaAI and HincII, were used on each successful amplification product according to the specifications provided by NEBioLabs. RFLP samples were then run out on a 2% agarose gel and the bands were analyzed to determine genetic differences between individuals of *S. oualaniensis*.

Nested PCR analysis was performed on samples 1,2, 4-8. A set of novel primers were designed for this study to selectively amplify segments of the COI sequence that differed between the normal specimens and the aberrant specimen with the goal of comparing the COI sequences of field samples to those of the already sequenced squid at a very fine resolution. A PCR mixture was first created which contained, for each sample, 2.5 μl 10x buffer, 2.5 μl dNTPS at 200 μM, 1.5 μl LCO 1490 [3'-GGTCAACAAATCATAAAGATATTGG-5'], 1.5 μl HCO 2198 [5'-TAAACTTCAGGGTGACC AAAAAATCA-3'], 1.5 μl "sthenoteuthis normal primer" [5' –AGTAATT AAAACAGATCAAGCAAACAGG-3'], 1.5 μl "sthenoteuthis ? primer" [5'-CAATTAATTTCATTACCACTATT-3'], 0.06 μl AMPLItaq polymerase, 1 μl sample DNA, and PCR grade H_2O added to obtain a 25 μl reacting volume. Samples were then covered with mineral oil to prevent evaporation and were run on a MJ PTC-150 PCR machine for 94˚C (2 min), followed by 34 cycles of 94˚C (30s), 50˚C (30s), and

72˚C (1 min) followed by a final extension at 72˚C (7 min). PCR products were then run-out on a 2% agarose gel impregnated with EtBr to ascertain differential amplification.

Results

18 samples were collected throughout this study between the latitudes of 19° 40.3' N and 3° 33.5' N and within several degrees of longitude of158º W (Figure 2). Of the 12 adult samples that were captured, 8 were positively identified as members of the photophore-bearing "typical" variety of *S. oualaniensis* (1, 2, 4, 5, 8 16, 17, 18) and 2 samples were positively identified as the photophoreless equatorial variety (6, 7). Morphological identification was based upon the presence or absence of a dorsal photophore, the number of ventral axes and the size of the specimen (Table 1). Larval squid could not be identified to morphological type, nor could two other samples (3, 15) for which only a partial specimen was obtained.

Restriction digest with BsaAI of the COI sequence yielded DNA fragments of approximately 400 and 220 base pairs for samples 1,2,4,5, 8, 11, 12 and 13. An identical digest of samples 6 and 7 yielded DNA fragments of approximately 200-250 base pairs and 150 base pairs (Figure 3). The BsaAI digests of samples 9 and 10 yielded inconclusive results, as did the digests of all samples with HincII.

Nested PCR produced DNA fragments of approximately 700 base pairs for samples 1, 2, 4, and 5. The same procedure produced DNA fragments of approximately 300 base pairs for samples 6 and 7 (Figure 4). Sample 8 produced no amplification product with nested PCR.

Discussion and Conclusion

Morphological analysis of *S. oualaniensis* samples confirms the long observed division of the species into at least two physical types, a photophoreless equatorial form and a larger, photophore bearing typical form (Nesis, 1993 after Clarke, 1965). The geographic distribution of the samples also matches previous observations of the distribution of these two types of *S. oualaniensis*.

A novel correlation between morphological type and COI sequence has also been observed. Genetic data suggests that all samples positively identified as being of the typical form (1, 2, 4, 5, 8 16, 17, 18) are genetically similar. All of these samples possess similarly sized COI fragments when subject to BsaAI analysis (~400/ 220 bp), as did all successfully digested larval samples (11, 12, 13). The BsaAI digest product of these samples also closely matched the calculated fragment size of previously sequenced samples of *S. oualaniensis* from around the Hawaiian Islands (394/ 220 bp). The observed genetic unity of all *S. oualaniensis* of the typical form is suggestive of an interbreeding metapopulation in the central and equatorial Pacific.

Interestingly, genetic assays of both samples of the equatorial type (6, 7) suggest that they are genetically similar to one another and significantly distinct from all samples of the typical variety. BsaAI digests produced similarly sized fragments for both samples, which were significantly different from the fragments produced for all other samples. The BsaAI fragment sizes of these two samples also matched the fragment sizes for the previously collected, genetically aberrant sample of *S. oualaniensis* (268, 220, 151 bp), which is presumed to be of the equatorial type (although a formal dissection has not yet been performed).

Furthermore, nested PCR produced similarly sized fragments for each specimen of the equatorial type. The size of these products matches the amplification product obtained for the genetically aberrant sample. This technique provides a very high-resolution comparison because almost the entirety of the 25 base pair primer must bind to a region specifically chosen for its variability. These results therefore provide strong evidence for genetic similarity both between the two samples of the equatorial type and between the equatorial samples and the previously collected aberrant sample. Associatively, these results suggest that the samples of the equatorial type, like the aberrant sample, have an 8% genetic difference from the typical type. This data therefore provides significant preliminary evidence for a population-level or even a species- level difference between the typical and equatorial varieties of *S. oualaniensis*. These findings disagree with the previous theory that the separation of the species into two forms stems from differential growth and maturation rates deriving from environmental factors (Nesis 1993 after Nesis 1977). However, the evidence supports the notion that the equatorial variety represents a nascent divergent species (Nesis, 1993) or even a fully diverged species (Nesis, 1993 after Nesis, 1985; Nigmatullin, 1983; Zuev, 1985).

There were a number of complications in the execution of this study. Larval samples 9 and 10 were not digested by BsaAI. It is possible that the restriction enzyme lacked a binding site because the samples are genetically distinct from both of the expected forms. It is also possible that the binding site was removed by a point mutation, which does not have larger ramifications for the genetic structure of the species. Another possibility is that contamination of the sample interfered with the ligation action of the enzyme. Genetic sequencing will be able to quickly resolve these possibilities.

All samples digested with HincII also produced inconclusive results. Because no DNA fragments were seen in the final assay, despite an affirmation of all PCR products in a previous gel, there is a high likelihood that the gel preparation was at fault, perhaps because of improper EtBr impregnation.

In addition, typical variety samples subjected to nested PCR analysis (1,2,4,5 and 8) did not produce a secondary amplification product. It is possible that these samples all differed significantly enough in the sequence of their COI regions from the sequenced sample for which the primer was designed to prohibit binding and subsequent amplification. It is also possible that primer degradation, human error, or a faulty primer synthesis (this primer was unable to be assayed before hand) may have led to a lack of secondary product. The lack of conclusive nested PCR results for the typical type samples makes it difficult to verify the degree of similarity between the specimens sampled in this study and the previously sequenced samples of the typical type. Genetic sequencing of the *S. oualaniensis* samples will almost undoubtedly help clarify this quandary and any other question calling for a finer look at the genetic sequences of these samples.

Understanding the population structure of *S. oualaniensis* is important for a number of reasons. If the equatorial form proves itself to be a nascent divergent species, it will serve as a fascinating example of sympatric speciation, since its range is completely within that of the typical form, and would merit further study for that reason alone. *S. oualaniensis* is also significant because of its huge ecological impact and its possible future importance for commercial fishing. It is the most abundant large squid in the Indo-Pacific region with an estimated biomass of 8-11 metric tons (Yatsu, 1998;

Young, 1998 after Nigmatullin, 1990). The biomass of *S. oualaniensis* in the Arabian Sea can reach incredibly high numbers of 12-42 tons per square kilometer in the months of November-January (Nesis, 1993). *S. oualaniensis* may also be the major nocturnal predator of mid-water micronekton in its habitat and is a major source of food for a host of vertebrate species (Young, 1998). As midwater ecosystems are becoming increasingly studied and commercial fisherman are intensifying their use of squid as a major source of income, a working knowledge of the population structure of *S. oualaniensis* may become a valuable asset.

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Tables and Figures

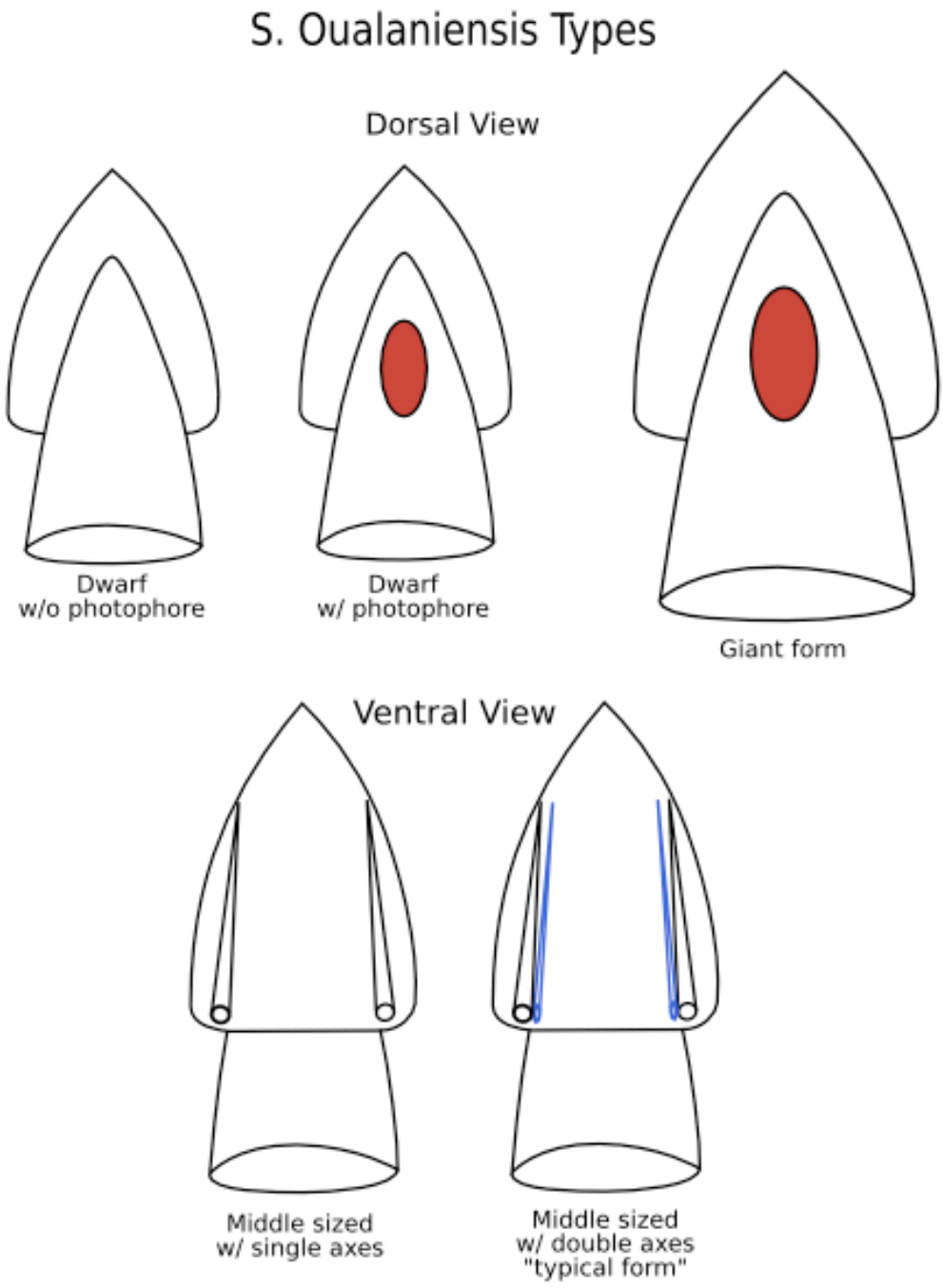

Figure 1: In the most highly divided form there are five morphological forms of *S. oualaniensis* . The dwarf from without a photopohore and the middle sized form with double axes were collected for this study.

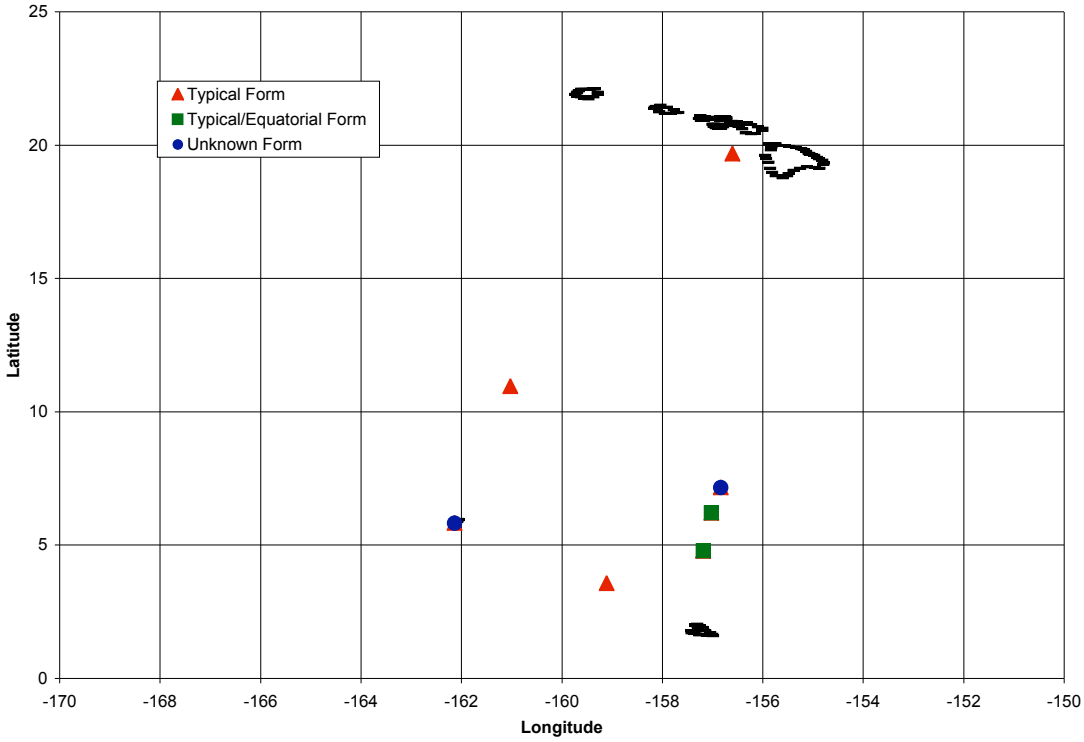

Figure 2: Map of the sampling area, from Hawaii to the Line Islands. The morphological type of the samples collected at each site, if known, is indicated in the legend.

Sample	Photophore	Axes #	Mantle Length (cm)	Mantle Width (cm)	Sex	Maturity	Type	Collection Site
1	present	2	17.2	11.3	female	adult	Typical	19° 40.3' N x 156° 36.1' W
2	present	2	13.3	9.8	male	adult	Typical	19° 40.3' N x 156° 36.1' W
3			6.4	5.1				7° 10.4' N x 156° 50.5' W
4	present	2	15.9	10.4	female	adult	Typical	6° 13.5' N x 157° 1.0' W
5	present	2	13.4	10	male	adult	Typical	6° 13.5' N x 157° 1.0' W
6	absent	1	11.5	6.7	female	adult	Equatorial	6° 12.6' N x 157° 1.5' W
7	absent	1	9.3	6.3	female	adult	Equatorial	4° 46.3' N x 157° 10.9' W
8	present	2	14.4	10.7	male	adult	Typical	4° 46.3' N x 157° 10.9' W
9			0.3			larval		4° 47.8' N x 157° 10.9' W
10			0.2			larval		4° 47.8' N x 157° 10.9' W
11			1.2			larval		3° 33.5' N x 159° 6.5' W
12			0.6			larval		3° 33.5' N x 159° 6.5' W
13			0.7			larval		3° 33.5' N x 159° 6.5' W
14			0.8			larval		5° 49.7' N x 162° 8.3' W
15						adult		10° 57.5' N x 161° 1.8' W
16	present	2	17.4	12.8	female	adult	Typical	10° 57.5 N x 161° 1.8' W
17	present	2	17.9	12.5	female	adult	Typical	10° 57.5' N x 161° 1.8' W
18	present	2	17.8	12.7	male	adult	Typical	10° 57.5' N x 161° 1.8' W

Table 1: Morphological characterists, morphological type, and coordinates of collection sites for each sample.

Figure 3: Results of BsaAI digest of COI with a 1 kb ladder. Samples 1, 2, 4, 5, 8, 11, 12, and 13 have fragments of approximately 400 and 220 base pairs in size. Samples 6 and 7 yielded fragments of approximately 200-250 and 400 base pairs. Samples 9 and 10 have undigested fragments of approximately 700 base pairs.

Figure 4: Results of nested PCR with 1 kb ladder. Samples 1, 2, 4, and 5 produced COI amplification products of approximately 700 base pairs with no secondary amplification. Samples 6 and 7 produced fragments of approximately 300 base pairs. Sample 8 produced no amplification product.