Analysis of population structure of O.cyanea from the Western Indian Ocean using analysis of microsatellite variation

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Final report for ProGeCo contract PE4/087/2011

Project title: **Analysis of population structure of *O.cyanea* from the Western Indian Ocean using analysis of microsatellite variation**

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Summary

The aim of the project was to screen genetic variation at 5 microsatellite DNA loci in a series of population samples of *Octopus cyanea* from Madagascar, to test for genetic differentiation between populations.

The objectives of the project were completed successfully, with three samples from Madagascar plus one outgroup comparison samples from Rodrigues being screened at 6 loci. In summary, the microsatellite DNA results indicate that there is little evidence for genetic differentiation among sites within SW Madagascar and that this area most likely comprises a single genetic population. There is evidence for genetic differentiation at a larger geographical scale across the SW Indian Ocean, but that this differentiation is of a low order of magnitude that is in keeping with some effective gene flow across the area typical of a widespread and abundant marine species with a dispersing larval stage.

Details of the work carried out

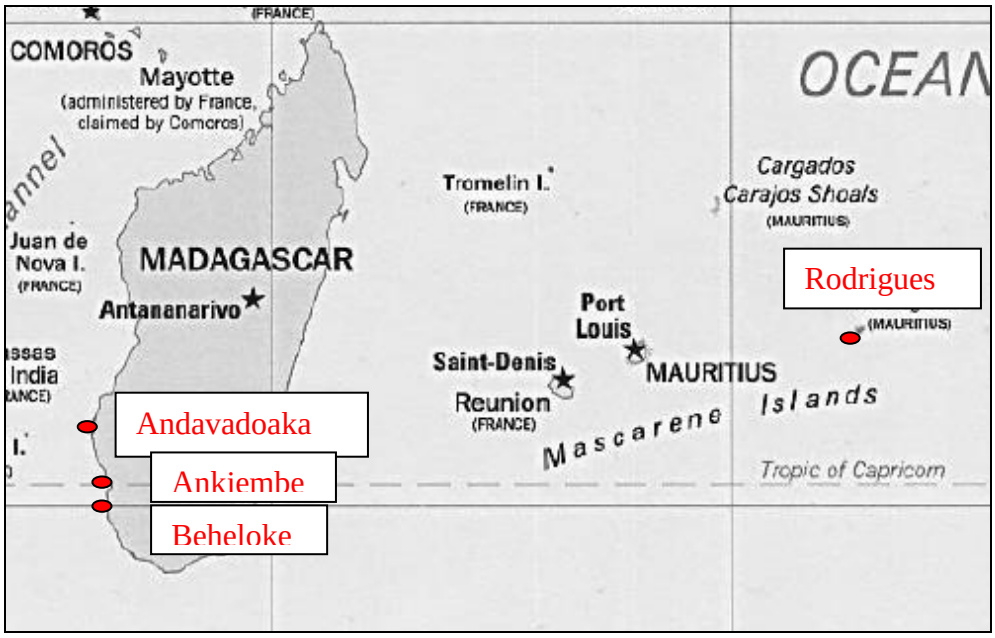
The project aim was to assess levels of genetic variation within, and differentiation between, population samples of *Octopus cyanea* from a range of sites across southwest Madagascar, using newly developed (contract PE4/087/2011) species-specific microsatellite DNA marker loci.

Sample Collection
A total of 240 individuals of *Octopus cyanea* were supplied by staff of Blue Ventures NGO, collected from three sites in Madagascar in August 2010, and a further sample of 80 individuals was collected by ReCoMaP staff from two sites in Rodrigues in February 2011 (Table 1, Fig.1), to act as an outgroup comparison against genetic variation within and between Madagascan populations. Samples were collected directly from fishermen or from fish markets: small pieces of arm tip were removed from fresh specimens, preserved in 70% ethanol and stored at room temperature.

Table 1. Location of sampling sites in Madagascar and Rodrigues

Location	**Sample size**
Andava (Andavadoaka), Madagascar	80
Ankiembe, Madagascar	80
Beheloke, Madagascar	80
North Rodrigues, Mauritius	40
South Rodrigues, Mauritius	40

Fig. 1: Location of sampling sites in Madagascar and Rodrigues.



Microsatellite marker screening

Total genomic DNA was extracted from each specimen using a modified CTAB / phenol / chloroform method (Winnepenninckx et al. 1993).

Each individual was screened for variation at 6 polymorphic microsatellite loci (Ocyn6, Ocyn19, Ocyn22, Ocyn31, Ocyn32 and Ov4), previously developed for *O. cyanea* (Shaw 2011) using Polymerase Chain Reaction (PCR) procedures. PCRs were carried out in a PTC-200 thermocycler (MJ Research) using the following conditions: 180 s at 95°C, then 45 cycles of 30 s at 95°C, 45 s at locus-specific annealing temperatures of 55-59ºC, followed by 30 s at 72°C. Each reaction mix contained 4µL template DNA, 2 mM $MgCl_2$, 0.5 µM forward primer and 0.5 µM of reverse primer, 0.2 mM dNTP mix, 1x reaction buffer [75 mM Tris-Hcl, 20 mM $(NH_4)_2SO_4$] and *Taq* polymerase (BIOTAQ, 5 U/µl) in a final reaction volume of 10 µL. Products were checked on a 2.0% agarose gel.

PCR products were then fluorescently labelled using M13 tailed primers labelled with PET, NED, VIC or 6FAM, and sized / genotyped on an AB3500 (Applied Biosystems) capillary DNA sequencer, using a LIZ600 size standard and GeneMapper software.

Data analysis

Genotypes at all pairs of loci were tested for genotypic linkage disequilibrium, and within loci within samples for deviation from Hardy-Weinberg outcrossing expectations, using Exact Tests with significance determined by a Markov chain method (GENEPOP v3.3; Raymond and Rousset 1995). Levels of genetic diversity within samples were estimated as the proportion of heterozygotes observed (H_o) and allelic richness (A, number of alleles observed corrected across all samples for the minimum sample size screened). Genetic differentiation among samples was analysed using Exact Tests of differences in allele frequencies (using GENEPOP) and F_{ST} (Wright 1978), which is a measure of genetic variation distributed between samples compared to that within samples, and varies from zero (identical gene frequencies) to 1 (samples fixed for different allelic forms). Levels of F_{ST}, estimated by θ (Weir and Cockerham 1984) both globally and pairwise between samples, were calculated and tested for significant departure from zero (no differentiation)

using permutation procedures within Fstat v2.9.3.2 (Goudet 1995). Where multiple tests were conducted, significance levels were adjusted according to a Bonferroni correction (Rice 1989).

Results

As the Rodrigues sample was drawn from two different sampling locations on the north and south sides of the island, allele frequencies in the two sub-samples were checked for significant differentiation to test whether they could be pooled to represent a single Rodrigues island population. None of the 6 loci displayed significant differences in frequencies between the two subsamples (6-locus combined exact test probability = 0.149), so the two subsamples were pooled for further analyses.
Across the 6 loci screened, 17 out of 60 tests displayed significant genotypic correlations in tests for linkage disequilibrium, and 12 tests remained significant after correction for multiple tests. However, all 12 highly significant linkage tests were associated with a single sample, Ankiembe: excluding this sample no locus pairs displayed significant linkage across all samples combined. As a result, all loci were considered to be independent genetic markers. The results for the Ankiembe sample indicate a sample-specific biological effect rather than a genetic linkage effect – see discussion below.
In tests for departure of genotype proportions from expectation under Hardy Weinberg equilibrium 9 out of 24 sample-by-locus tests were significant at $P<0.05$, and 7 remained significant after Bonferroni correction for multiple tests. As with the linkage disequilibrium, 4 of these highly significant tests were due to the Ankiembe sample: the remaining tests were not associated consistently with any particular locus or sample.

Tests for genetic differentiation in gene frequencies indicated small but significant differences among the samples, with an $F_{ST} = 0.005$ ($P=0.0002$) and exact test $P<<0.001$. Examination of the tests for pairwise differentiation between individual samples (Table 2) reveals the main source of the global differentiation to be due to highly significant results between the Ankiembe sample and all other samples. No significant differences are indicated between the Andavadoaka and Beheloke samples. Genetic differences between the Andavadoaka and Rodrigues samples are low and near the margin of significance, and between Beheloke and Rodrigues are also low but significantly different from zero for both F_{ST} and exact tests.

Table 2. Estimates of genetic differentiation (F_{ST} below diagonal, Exact Test probability above diagonal) between samples of *O. cyanea*. Tests for F_{ST} significantly greater than zero: * = p<0.05; ** = p<0.01; values in bold remain significant after Bonferroni correction for multiple tests (p<0.008). See Table 1 and Fig.1 for sample location.

	And	Ank	Beh	Rod
And		**<0.001**	0.112	0.051
Ank	**0.006****		**<0.001**	**<<0.001**
Beh	0	**0.004****		**0.002**
Rod	0.002	**0.013****	0.005*	

The within-sample genetic tests on the Ankiembe sample suggested that something very odd was occurring within this sample. Closer inspection of the multi-locus genotypes across all individuals revealed that a number of genotypes were represented multiple times: 13 6-locus genotypes represented between 2 and 5 times each. The most parsimonious explanation for the occurrence of replicate multi-locus genotypes is that during sampling more than one arm tip was included from single individuals: the alternative is that *O.cyanea* has asexual reproduction (i.e. generates clones), which has never been reported for a cephalopod species in the literature.

Assuming that the replicate genotypes do represent multiple sampling of the same individual, the sample was subsequently cleaned of all replicates (i.e. one example of each was left in), and put through the analysis again. After cleaning of the sample genotypes the Ankiembe sample displayed only one locus with significant departure from Hardy-Weinberg out crossing equilibrium, although did still display 4 highly significant tests for linkage disequilibrium – it is possible therefore that one or two genotypic errors may still be present in this sample data, and so the sample results should be viewed with caution until a new sample definitely free of replicate sampling can be analysed.

After cleaning the Ankiembe sample, tests indicated much lower values of genetic differentiation than produced by the uncleaned sample but still with significant differences in allele frequencies between the Ankiembe sample and both the Andavadoaka and Beheloke samples (exact tests P = 0.020 and 0.017 respectively), although the F_{ST} values were very low and not significantly different from zero (F_{ST} = 0.003 and 0.001 respectively) – see Table 3. Genetic differences between the Ankiembe and Rodrigues samples are low but significantly different from zero for both F_{ST} (0.010, P<0.01) and exact tests (P<0.001).

Table 3. Estimates of genetic differentiation (F_{ST} below diagonal, Exact Test probability above diagonal) between samples of *O. cyanea* **after cleaning of replicate genotypes from Ankiembe sample**. Tests for F_{ST} significantly greater than zero: * = p<0.05; ** = p<0.01; values in bold remain significant after Bonferroni correction for multiple tests (p<0.008). See Table 1 and Fig.1 for sample location.

	And	Ank	Beh	Rod
And		0.020	0.112	0.051
Ank	0.003		0.017	**<0.001**
Beh	0	0.001		**0.002**
Rod	0.002	**0.010****	0.005*	

Interpretation

Screening of the newly developed genetic markers for *Octopus cyanea* confirmed that these microsatellite DNA loci possessed suitable levels of allelic variation for population genetic analyses and that the loci could be used as independent (i.e. non-linked) markers with no obvious technical issues.

There is no evidence for genetic differences between the Andavadoaka and Beheloke samples, suggesting no substantial genetic differentiation across this area of SW Madagascar and no evidence for major barriers to gene flow and population connectivity. These sites could be regarded as belonging to the same genetic population. Although there is marginally significant ($P<0.05$, but becomes non-significant if corrected for multiple tests at $P < 0.008$ – see Table 3) genetic difference indicated by the exact test results for the Ankiembe versus the other two Madagascar sites, the F_{ST} values are low and non-significant. Given the technical problems associated with the screening of the Ankiembe sample, the conservative interpretation would be assume that the Ankiembe site also has very little (and probably insignificant) genetic differences from the Andavadoaka and Beheloke sites. These results suggest therefore that the *O. cyanea* population across SW Madagascar largely represents a genetically homogeneous population with no substantial evidence for localised genetic differentiation or isolation of local populations. Levels of genetic diversity within samples is similarly high for all three Madagascan localities (allelic richness ranging from 5.8-6.9 at locus Ov4 to 17.5-18.9 at locus Ocyn6, and gene diversity ranging from 3.4-9.8 at the same loci), indicating that all three sites have similarly large and diverse populations.

Surprisingly low levels of genetic differentiation (<1% of genetic variation distributed between sites) were detected between the SW Madagascar sites and the outgroup sample from Rodrigues, given the large distance of open ocean between these areas. When the Andavadoaka and Beheloke Madagascar samples are pooled and tested against Rodrigues, genetic differentiation is low ($F_{ST} = 0.003$) and of marginal significance ($P=0.046$). When all three Madagascar samples (i.e. including the cleaned Ankiembe sample) are pooled and tested against Rodrigues, genetic differentiation is slightly higher ($F_{ST} = 0.005$) but still of marginal significance ($P=0.050$). These results suggest that the Rodriges population is genetically divergent from the Madagascar population, and so not exchanging migrants between the two areas (i.e. gene flow through larval dispersal) on a large scale or regular basis. The very low level of differentiation suggests that the populations of these two areas have diverged only recently, or that occasional gene flow does occur either directly or via intermediate populations (Mauritius and Reunion?). Prevailing current flows across the region from east to west, and the biology of the species (i.e. pelagic larval stage), would support the potential for larval gene flow from Rodrigues to Madagascar – future studies employing further molecular marker loci should be able to address this question of unidirectional gene flow. Genetic diversity within the Rodrigues sample is lower than in the Madagascan samples at all loci screened (allelic richness = 3.5 - 16.6, compared to 6.4 – 18.3), indicating that effective population size is lower in Rodrigues and/or that gene flow into Rodrigues from other areas is probably restricted compared to that into Madagascar (supporting the unidirectional gene flow concept).

In summary, the microsatellite DNA results indicate that there is little evidence for genetic differentiation among sites within SW Madagascar and that this area most likely comprises a single genetic population. There is evidence for genetic differentiation at a larger geographical scale across the SW Indian Ocean, but that this differentiation is of a low order of magnitude that is in keeping with some effective gene flow across the area typical of a widespread and abundant marine species with a dispersing larval stage.

ANNEX II

RESEARCH CONTRACT
TERMS OF REFERENCE

Analysis of population structure of *O.cyanea* from the Western Indian Ocean using analysis of microsatellite variation

Regional Programme for the Sustainable Management of the Coastal Zones of the Countries of the Indian Ocean (ReCoMaP) (9.ACP.RSA.020)

1. Introduction to ReCoMaP

In most coastal zones of the SWIO, human population growth, economic development, and concomitant increases in unregulated natural resource use are leading to severe ecosystem deterioration and damage to ecological productivity. This is having the effect of intensifying coastal resource use competition and creating conflicts between the stakeholders that exploit the increasingly crowded coastal zone. However, coastal zones are also dynamic and complex areas, both environmentally and in terms of their use by human societies. This complexity demands the development of effective *integrated* natural resource-use management systems i.e. Integrated Coastal Zone Management (ICZM).

The Regional Programme for the Sustainable Management of the Coastal Zones of the Countries of the Indian Ocean (ReCoMaP) is a programme of the Indian Ocean Commission (IOC) financed by the European Union (EU). It is a five-year programme, which aims to enhance sustainable management and conservation of natural coastal and marine resources with a view to contributing to poverty alleviation among coastal populations. The beneficiary countries are Comoros, Kenya, Madagascar, Mauritius, Seychelles, Somalia and Tanzania. ReCoMaP is implemented through a Regional Coordination Unit (RCU) based in Quatre Bornes, Mauritius.

ReCoMaP works with partners in the SWIO to support and facilitate ICZM capacity development towards national and regional institutional arrangements that can promote integrated decision-making and, ultimately, effective Integrated Coastal Zone Management.

This Research Contract relates to ReCoMaP's Result Area 1 - to contribute to *the sustainable management of coastal and marine biodiversity* in the WIO region.

2. Background to the Research Project

Octopus cyanea is a widespread and abundant species throughout the Indian Ocean (and Pacific Ocean), and forms the basis of important subsistence and commercial fisheries throughout the region. In order to formulate fisheries management, it is important to gather information on the structuring of local populations within regions and inter-connectivity of populations between regions. Population genetic approaches, using DNA-based markers, have proven to be valuable in providing data on genetic structuring and connectivity of populations, as well as on levels of genetic biodiversity and gene flow (migration) among populations. At present no population genetic information is available for *O.cyanea*, certainly not within the SW Indian Ocean region, and so it is difficult to assess the levels of genetic diversity within local populations, the geographical

scale of local interbreeding populations, or the degree of inter-connectivity between populations across the region.

Sample sites will be selected by ReCoMaP staff in order to address questions relevant to local fisheries management priorities, and samples provided to the Contractor.

3. Tasks for the Contractor

A series of population samples, collected from across the region, will be screened for genetic variability within, and genetic differentiation between, populations. A suite of 5 *O.cyanea*-specific microsatellite DNA marker loci, newly developed in our laboratory, will be PCR amplified and screened for individual variation.

Sample sizes of up to 100 individuals per site will be screened to provide statistically robust estimates of diversity and differentiation. Samples from four sites, as selected by ReCoMaP staff, will be screened for variation.

For each *O.cyanea* individual, ethanol-preserved arm tissue will be digested and total genomic DNA extracted using standard commercial kits. DNA primers specific to the selected marker loci will be tested against the sample DNA and optimized for optimal genotyping clarity (to take account of sample condition). Each individual will be genotyped at a minimum of 5 marker loci, using an AB3500 Genetic Analyser.

The assembled genotype dataset will be analysed using standard population genetic procedures and programmes (e.g. GENEPOP, FSTAT, FreeNA) to check for departures from linkage and Hardy Weinberg equilibrium, to assess levels of within-sample diversity, and to test for between-sample differentiation (to estimate levels of gene flow).

A report will be produced detailing the analyses conducted, the results obtained, and interpretation with regard to population connectivity including a 1-page Policy Brief on the implications of the findings.

4. Timing and Duration

The Research Contract will be implemented over a period of 2 months, with an indicative start date of 1st May, 2011.