Characterization of the Mitochondrial DNA Control Region of the Wahoo, *Acanthocybium solandri*, from the Northcentral Gulf of Mexico and Bimini, Bahamas

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ABSTRACT

Wahoo, *Acanthocybium solandri*, rank among the most coveted of fish sought by offshore recreational anglers in many parts of the world and are the basis of important commercial fisheries. Little information is available on the biology and life history of the wahoo and no information exists on the molecular genetics of this pelagic, circumglobally distributed species. Our preliminary research consisted of a molecular characterization of the mtDNA control region, including structure and sequence of the flanking tRNA genes. Total DNA was isolated from ten wahoo tissue samples: five from the northcentral Gulf of Mexico and five from Bimini, Bahamas. Universal primers produced a fragment of approximately 1830 base pairs (bp) containing the tRNAs for proline, threonine, and phenylalanine plus the entire control region of one fish from each site. The fragments were gel purified for direct sequencing and species specific primers were designed in the tRNAs immediately flanking the control region and used in subsequent amplifications. The control region of the ten wahoo ranged in size from 886 bp to 892 bp. One termination activation sequence (TAS I), three conserved sequence blocks (putative CSB I, CSB II, CSB III), five repeats, and two inverted repeats were identified. The 5' end of the control region contained the greatest sequence variability with approximately 100 variable bases and 3 indels within the first 330 bp. Multiple restriction sites for HinfI, RsaI, and SspI were identified that could be used to distinguish each fish as a unique haplotype by RFLP analysis. Results provide a characterization of the wahoo mtDNA control region for possible use in future investigations.

KEY WORDS: *Acanthocybium solandri*, genetics, mitochondrial DNA

INTRODUCTION

The wahoo, *Acanthocybium solandri*, is a pelagic species found worldwide in tropical and warm temperate oceanic waters (Collette and Nauen 1983). Wahoo typically occur a considerable distance offshore, particularly around islands, pinnacles, ancient coral heads, and offshore banks. Attracted to current edges and temperature breaks, wahoo also occasionally occur nearshore

along reef edges and walls. Common names for *A. solandri* depend on geographic location and include wahoo, ocean barracuda, tigerfish, kingfish, pride of Bermuda, peto, ono, oahu, and robalo.

Wahoo commercial landings from the western central Atlantic, including the Gulf of Mexico, increased from an average of 2.9 metric tons per year between 1974 and 1978 to 164 metric tons in 1992, and steadily decreased to 102 metric tons in 1996 (Mahon 1996). National Marine Fisheries Service (1999) data indicated that the wahoo landings in the Gulf of Mexico and Atlantic (off US states only), also peaked in 1992 and fluctuated between 1993 and 1998, with an overall decrease. Increased recreational fishing in U.S. and Caribbean waters has put additional fishing pressure on this species. There is very little information available on the biology and life history of the wahoo, and there is no published information on molecular genetics. With the exception of tag and release data, little information exists concerning the spatial limits of the pelagic habitat, or how this relates to the amount of genetic exchange actually occurring among populations of large, oceanic fishes (Rosel and Block 1996).

In this study, we used mitochondrial DNA (mtDNA), because it accumulates mutations at a rate up to ten times faster than the nuclear genome (Meyer, 1993). Within the mitochondrial genome, there are 13 genes that code for proteins, two that code for ribosomal RNAs (rRNA), and 22 that code for transfer RNAs (tRNA). Also, there is a noncoding region, termed the control region, that accumulates mutations two to five times faster than the rest of the mitochondrial genome (Meyer 1993). Since this is the most rapidly changing region of DNA in the cell, it can be useful in assessing population structure. This region, also referred to as the "D-loop" or "displacement loop region," contains conserved motifs and the sites of initiation for both heavy-strand replication and heavy- and light-strand transcription (Chang and Clayton 1987, Clayton, 1991a, 1991b; Digby et al. 1992).

The purpose of our study was to obtain the entire sequence of the tRNAs flanking the control region, proline (tRNA-pro) and phenylalanine (tRNA-phe), and use this information to design species specific primers to direct sequence and characterize the control region of wahoo.

MATERIALS AND METHODS

Wahoo were collected from the northcentral Gulf of Mexico, USA and off the coast of Bimini, Bahamas. Tissue was excised and fixed in SED buffer (250 mM EDTA, pH 7.5, 20% DMSO, 3.42 M NaCl). Total genomic DNA was extracted from each sample using a procedure modified from Taggart *et al.* (1992), quantified using fluorescence spectrophotometry (Gallagher 1994) and adjusted to concentrations of 100 ng/μL using 1 mM Tris, pH 8.5.

The universal primers CB3R (5' CACATTCAACCAGAATGATATTT 3';

Palumbi, 1996) and 12SA-H1067, referred to in the present work as 12SAR, (5' ATAATAGGGTATCTAATCCTAGTT 3'; Martin et al. 1992) were used to amplify portions of the cytochrome b gene and 12S rRNA from four fish (two from each site), the tRNAs flanking the control region, and the entire control region by PCR (Figure 1). Appropriate PCR-products were gel-purified (QIAquick Gel Extraction Kit, Qiagen, Inc.), quantified, and sequenced. Species specific primers in the tRNA-pro (WahPro, 5' CCTACCCCTAACTCCCAAAGCTAG 3') and tRNA-phe (WahPhe, 5' GCTTTCTAGGGCCCATCTTAACAT 3') were designed from sequence information and used to amplify the entire control region from additional fish by PCR (Fig. 1). PCR amplification was conducted for replicate 50 μL reactions containing 200 ng template DNA, 1.5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphates (Promega, Inc.), 0.4 μM of each primer, and 3.5 units of *Taq* DNA polymerase with 10x PCR buffer (Amersham Life Science). Cycling parameters utilizing wahoo primers were a 3 min denaturation at 94°C, followed by 35 cycles consisting of 0.5 minutes at 94°C, 0.5 min at 58°C, and 1.5 minutes at 72°C, and a final elongation of 7 minutes at 72°C. Appropriate PCR-products were gel-purified, quantified, and sequenced. All sequencing was conducted at the University of Maine DNA Sequencing Facility on an ABI model 373A automated sequencer.

In an effort to verify that the PCR product obtained using the species specific primers resulted from amplification of mtDNA and not a nuclear pseudogene, a nested PCR using control region primers from a previously published study (Lee et al. 1995) was conducted. In this procedure, gel purified products obtained from the amplification with CB3R and 12SAR were used as template for a second reaction with the afore mentioned internal primers designated as "A" 5' TTCCACCTCTAACTCCCAAAGCTAG 3' and "G" 5' CGTCGGATCCCATCTTCAGTGTTATGCTT 3' (Figure 1).

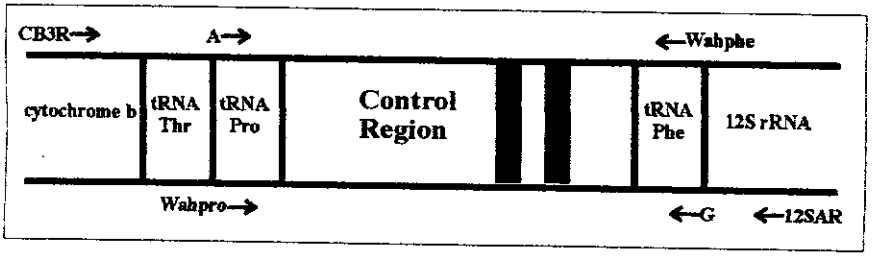


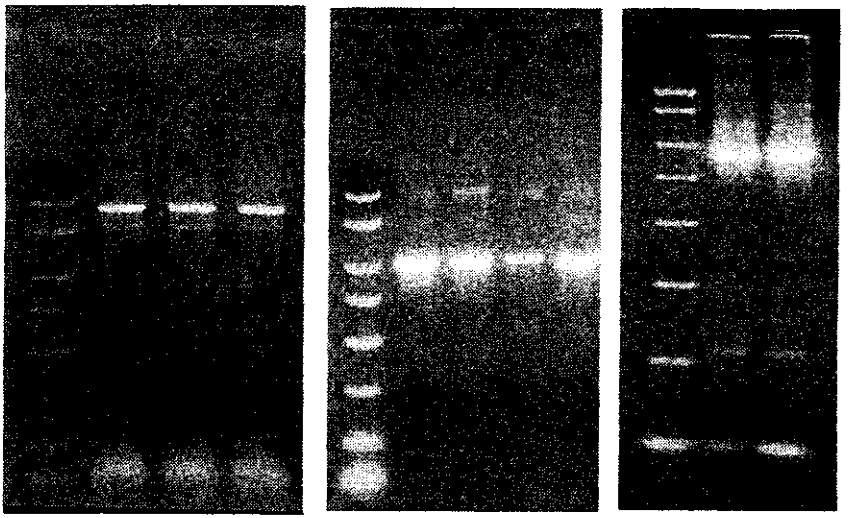
Figure 1. A view of a portion of the piscine mitochondrial genome. Arrows indicate the relative position of primers employed to amplify the control region of the wahoo, *Acanthocybium solandri*.

Secondary structures of the tRNAs threonine (thr), pro, and phe were elucidated using Sean Eddy's Lab tRNAscan-SE Search Server (Lowe and Eddy 1997). DNA sequences were imported into OMIGA, ver. 1.1 (Oxford Molecular Ltd., Oxford, England), a multiple sequence editor, and aligned with CLUSTALW (Higgins and Sharp 1988) using default settings. OMIGA was also employed to search for restriction endonuclease digestion patterns and assign each sequence to a distinct haplotype.

RESULTS

The universal primers CB3R and 12SAR produced a DNA-fragment of approximately 1800 base pairs (bp) in the four initial samples (Figure 2a). The complete control region sequence was obtained from two fish, one from each sampling site and deposited in GenBank (accession numbers AF197149 and AF197152). These sequences were compared against sequences deposited at the National Center for Biotechnology Information (NCBI) using the NCBI's BLAST WWW Server (Basic Local Alignment Search Tool; Altschul *et al.*, 1990), and the closest matches were obtained with the mtDNA control region of several species of fish including *Etheostoma zonale*, *Gymnocephalus cernuus* and *Gymnocephalus schraetser*. The sequence and structure of the flanking tRNAs were also identified in all four samples (Figure 3). This sequence information was utilized to design species specific primers located in the tRNA-pro (WahPro) and the tRNA-phe (WahPhe). These primers produced a DNA-fragment of approximately 950 bp from eight fish (Figure 2b). BLAST searches indicated these sequences were the mtDNA control region. Nested PCR of the gel-purified CB3R/12SAR product with the primers A and G produced a DNA-fragment of approximately 950 bp (Figure 2c), as anticipated.

The control region of ten fish ranged from 886 to 892 bp (GenBank accession numbers AF197149-58), with a consensus sequence of 897 bp (Figure 4). The consensus sequence contained 128 transitions, 22 transversions, and 20 insertions/deletions (indels). One termination associated sequence (TAS) was identified: TAS-I, located in the tRNA-pro (Figure 4). Two conserved sequence blocks (CSB), CSB-II and CSB-III, were identified at the 3' end of the control region, and a putative CSB-1 was identified at the 5' end (Figure 4). A hypervariable region, containing 100 variable nucleotides and three indels in 320 bp, was identified at the 5' end of the control region (Figure 4). Five direct repeats were identified, as well as, two indirect repeats (Table 1 and Table 2). Eight of the 30 restriction endonucleases used in computer-simulated digestions revealed polymorphism (Table 3a). Three of these eight (Hinf I, Rsa I, and Ssp I) revealed several morphs that were unique to each sampling site (Table 3b).



A. B. C.

Figure 2. Agarose gels of the mitochondrial DNA control region of the wahoo, *Acanthocybium solandri*. The control region was PCR amplified using the following primer sets: (A) CB3R/12SAR, (B) WahPro/WahPhe, and (C) A/G (see Materials and Methods for details). Agarose Gel Ladder sizes: 2000, 1500, 1000, 750, 500, 300, 150, and 50 base pairs (Amresco PCR marker, Amresco, Inc.).

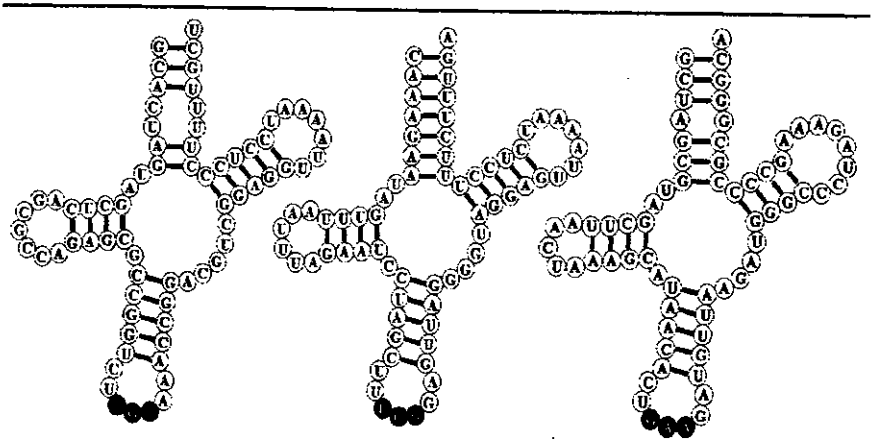


Figure 3. Sequence and structure of the mitochondrial tRNAs (from left to right), threonine, proline, and phenylalanine from the wahoo, *Acanthocybium solandri*. Black circles represent the anticodons.

TAS I

CTAAATTAAA CTATTCTTTG PRO

GCGACGCTCA CGCAATATAy AATAATATGT CTGTGAAGTA 40
CATrTATGTA TTTACACCAT AyATTTATAT TAAyCATAyw 80
AwTrCATATm TdGTACTCAw GGACATrvry ATGyTdAAyr 120
TACATryCTA TTCTyGrrTA yAATryTyrT ATTGCyyGAy 160

CSB I

yyyAyTAGrA ryTAyATryG **TACATAAACC** AyAATrTGTA 200
yTArryyyyrk TayAyrhyrC TGATyTCwrG GayAGAyGAr 240
ATTTAAGAyC TAGCACGAAA ACGTCCATrG rCAAATAyAT 280
ACCAAGyAyT CAryAyCArr ATGrryyyny dyAAATyTTA 320
ATGyAGTAAG ArCCTACCAT CAGTTGATTC CTTAATGCAT 360
ACGGTTmTTG AAGGTGAGGG rCAATAryhG TGrGGGTyrC 400
ACyyAGTGAA ywwTyCyyGG GCATTkGGGT TCCTAATTTC 440
AGGTCCATyT rAyTTGAywy AyTCCyCATT CTTTCCTTGA 480
CGCTGGCATA AGTTAATGGT GGAGTACATT rGACTCGTTA 520
CCCACCATGC CGrGCryTCT TTCCATCGGG CATrGGkTTT 560
yyyyTTTTkG GTTyCyTywC AyTTGrCATC CCAGAGTGCA 600
CACrAAGAAT AGyTrAyAAG GTTGAACATT TCCTTGCTTG 640
CAGrGTAAAT rTCATGAAGG GTGGAAAGAC TTTACrykAA 680
rrArCCACAy ryTyGGATAT CAAGAGCATA ArTGAAATAT 720
yACTCGAArA ATATyTAAGG TGCCCCCTCT CGGCTTTTGC 760

CSB II

GCGT**TAAACC CCCCTACCCC** CCTAAAyTCG TGAAATATyT 800

CSB III

AACACTCC**TG AAAACCCCCC GTAAACA**GGA AAACTCTCGA 840
GTGGGGTATT TTATAGCCCA AAACGTATCT ATTTACATTA 880
TTGTAAATAT TGCGTAT 920

Figure 4. Consensus sequence of the mitochondrial DNA control region of the wahoo, *Acanthocybium solandri*. Termination associated sequences (TAS) and conserved sequence blocks (CSB) are in bold text. The 320 base pair hypervariable region is underlined. The top line is the 3' end of the tRNA proline.

Table 1. Direct repeats located in the mitochondrial DNA control region of the wahoo, *Acanthocybium solandri*. Repeats were identified from the consensus sequence of ten individuals; five from the northcentral Gulf of Mexico and five from Bimini, Bahamas. The minimum repeat length was seven base pairs (bp).

Number	Fragment from base	Repeated from base	Size of repeat (bp)	Repeat sequence 5' 3'
1	37	503	7	AGTACAT
2	49	869	8	TATTTACA
3	472	629	8	TTTCCTTG
4	766	812	9	AAACCCCCC
5	768	776	8	ACCCCCCT

Table 2. Indirect repeats located in the mitochondrial DNA control region of the wahoo, *Acanthocybium solandri*. Repeats were identified from the consensus sequence of ten individuals; five from the northcentral Gulf of Mexico and five from Bimini, Bahamas. The minimum repeat length was eight base pairs (bp).

Number	Fragment from base	Repeated from base	Size of repeat (bp)	Repeat sequence 5' 3'
1	10	887	9	ACGCAATAT
2	537	662	8	TCTTTCCA

Table 3a. Restriction endonucleases employed in a computer-generated restriction digestion of the mitochondrial DNA control region of the wahoo, *Acanthocybium solandri*. The procedure was conducted on each sequence for ten individuals; five from the northcentral Gulf of Mexico and five from Bimini, Bahamas.

Alu I**	Hpa I	Sac I
BamHI	Kpn I	Sal I
Bgl I	Mlu I	Sca I**
Bgl II**	Msp I**	Sfi I
Dra I	Nco I**	Sma I
EcoRI	Not I	Sph I
EcoRV	Nru I	Ssp I**
Hae III	Pst I	Taq I*
Hind III	Pvu II	Xba I
Hinf I**	Rsa I**	Xho I*

* denotes the presence of cuts without polymorphism between the ten sequences

** denotes polymorphism between the ten sequences

Table 3b. Unique haplotypes observed with respect to geographic location in a computer-generated restriction digestion of the mitochondrial DNA control region of the wahoo, *Acanthocybium solandri*. These haplotypes were generated from analysis of each sequence for ten individuals; five from the northcentral Gulf of Mexico and five from Bimini, Bahamas.

	Hinf I					Rsa I								Ssp I				
	A	B	C	D	E	A	B	C	D	E	F	G	H	A	B	C	D	E
Location																		
Gulf	3	0	0	1	1	1	2	0	0	0	0	1	1	0	3	0	1	1
Bimini	2	2	1	0	0	1	0	1	1	1	1	0	0	4	0	1	0	0

DISCUSSION

The universal primers, CB3R and 12SAR, when used to amplify wahoo template, produced an approximately 1800 bp piece of DNA having significant similarity to the mtDNA control region and flanking tRNAs of numerous fish when compared with a BLAST search. After species specific primers were developed in the flanking tRNAs, a fragment of the expected length (approximately 950 bp) was produced showing high similarity to both the corresponding sequence of the original fragment and the mtDNA control region of other fish when compared using a BLAST search. The fact that the PCR product obtained with the species specific primers was the predicted length, alone, was a direct indication the designed primers were amplifying the desired portion of the mtDNA (Palumbi 1996). An additional precaution was taken to exclude the possibility that the PCR products obtained with the species specific primers were in fact mtDNA and not nuclear pseudogenes similar to those observed in other species of marine organisms such as sea urchins, crabs, and corals (Jacobs et al. 1983, Palumbi 1996). Following the concept of "long PCR" (Cheng et al. 1994a, 1994b), the 1800 bp piece of DNA produced using the universal primers CB3R and 12SAR was used as template for a second internal PCR with previously published fish mtDNA primers located in the tRNAs for proline and phenylalanine. The internal PCR product produced a band of the expected size, confirming the piece of mtDNA amplified was the desired control region sequence.

The length of the control region in wahoo was 886-892 bp which is comparable to the control region of other fish species. Lee et al. (1995) found the control region of 27 species of fish ranged from 856-1500 bp. In that study, cichlids and gadids contained the shorter control regions, and the control regions of pleuronectids were longest. Control region structure, a central conserved region flanked by more variable segments, was also similar to that reported for other fish species (Buroker et al. 1990, Johansen et al. 1990, Digby et al. 1992,

Lee et al. 1995). We identified a TAS-I, as well as, a putative CSB-I, a CSB-II, and a CSB-III. The nucleotide sequences of these regions are similar to those of numerous fish (Table 4). CSB-I was conserved in the ten wahoo sequenced, but its location was atypical from other species of fish (Digby et al. 1992, Wilson et al. 1997) and, thus, was reported as putative.

Table 4. Comparative alignment of termination associated sequence (TAS) and conserved sequence blocks (CSB) identified in the wahoo, *Acanthocybium solandri*, and other fish species.

TAS-I		
	Wahoo	AAATTAAACTATT
	Trout[1]	AAATTAAACTACC
	Sturgeon[2]	ACATTAAACTATT
Putative CSB-I		
	Wahoo	ACATA
	Trout	ACATA
	Snook[3]	ACATA
CSB-II		
	Wahoo	TAAACCCCCCTACCCCC
	Trout	TAAACCCCCCTACCCCC
	Sturgeon	-AAACCCCC-TACCCCC
CSB-III		
	Wahoo	TG-AAAACCCCCCGTAAAC-A
	Trout	TGTTAAA-CCCC—-TAAACCA
	Swordfish[4]	TG-AAAA-CCCCC

[1]Digby et al. 1992; [2]Buroker et al. 1990; [3]Wilson et al. 1997; [4]Rosel and Black 1996

We also identified a 320 bp hypervariable segment at the 5' end of the control region that contained 100 variable nucleotides and three indels. Similar hypervariable segments have been observed near the 5' end of the control region of other fishes (Lee et al. 1995, Rosel and Block 1996, Garber 1999). Rosel and Block (1996) found a high level of polymorphism in the control region of swordfish collected from three different ocean basins. They concluded a DNA region less variable than the control region might be necessary to study the population structure of fish from the same ocean basin. Although, to arrive at such a conclusion with wahoo, would require the sequencing of more fish and an outgroup.

Eight of 30 restriction endonucleases produced polymorphic fragments and

three of these enzymes; Hinf I, Rsa I, and Ssp I separated the ten wahoo sequences into unique haplotypes. A rapidly evolving region such as the mtDNA control region has the potential for use as a stock identification tool and/or a stock separation tool. If a large number of unique mtDNA haplotypes are observed, restriction endonucleases can be a suitable molecular tool to identify a stock, as well as, to evaluate the impact of a stock enhancement effort on reproduction and recruitment in subsequent generations (Gold and Richardson 1991; Gold et al. 1997, Wilson et al. 1997). Again, further wahoo samples need to be sequenced to arrive at such a conclusion.

Increased pressure has been placed on the wahoo due to its rising importance in commercial and sport fisheries. Therefore, obtaining information on its biology, life history, and genetics is essential to prevent a decrease in stocks beyond a sustainable level and the associated loss of genetic diversity. This study provides initial molecular genetic data that will prove useful in future genetic research.

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