

International Whale Shark Conference 2008

THE CONTROL REGION OF MITOCHONDRIAL DNA AS A SPECIFIC MOLECULAR MARKER OF WHALE SHARK (*Rhincodon typus*).

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Rhincodon typus (Smith, 1829)

Phylum Chordata

Subphylum Vertebrata

Class Chondrichthyes

Subclass Elasmobranchii

Order Orectolobiformes

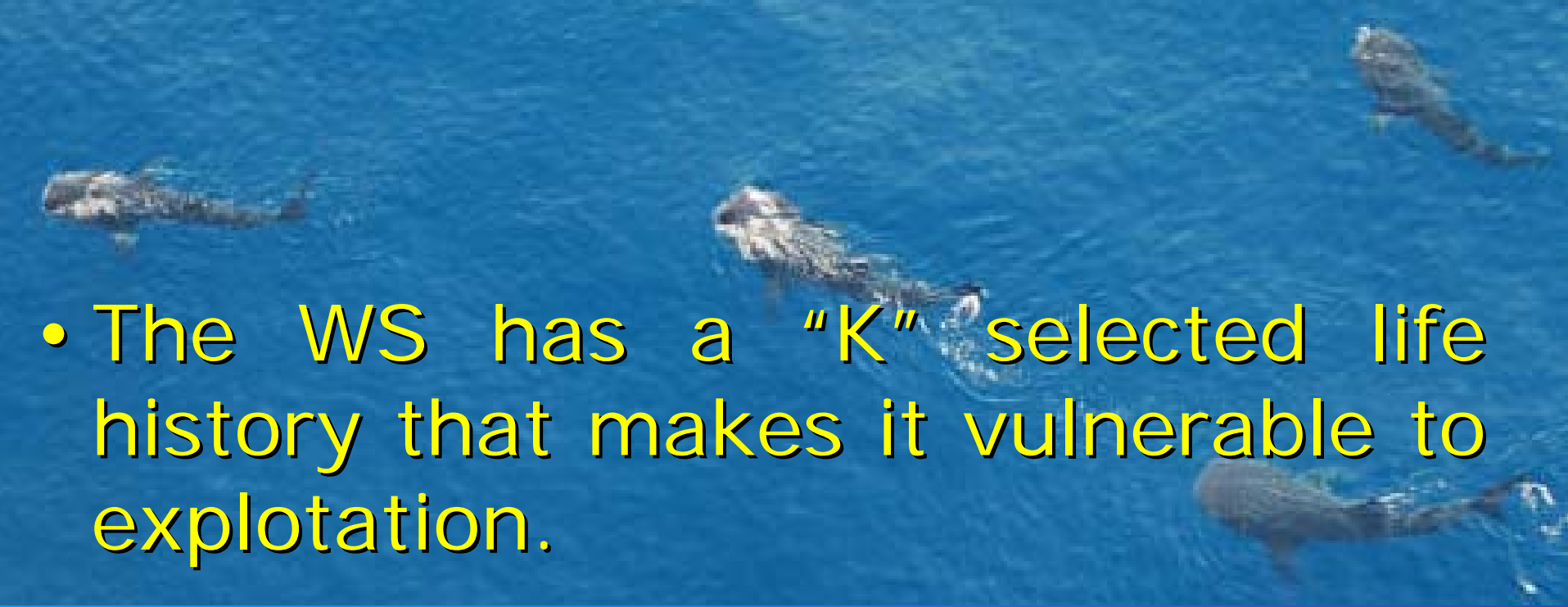
Family Rhincodontidae



Orectolobiformes

- Hemicyllidae Gill, 1862
- Orectolobidae Jordan y Fowler, 1903
- Brachaeluridae Applegate, 1972
- Parascyllidae Gill, 1862
- Ginglymostomatidae Gill, 1862
- Stegostomatidae



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- The WS has a “K” selected life history that makes it vulnerable to exploitation.

- The WS is a specie that has suffered because of fisheries.

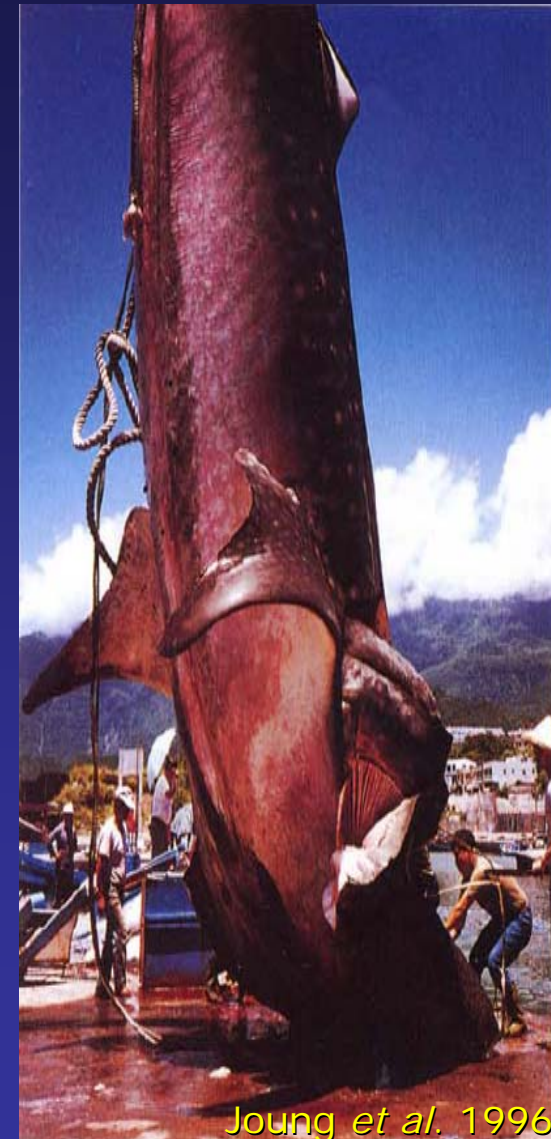


FISHERIES

- Maldives, China, Taiwan, India, Pakistan, Japan, Indonesia, Philippines and Senegal

NATIONAL PROTECTION

- In Australia it has total protection.
- In the USA it has total protection.
- In Belize it is protected since 2000 (Decree No.68).
- In Honduras it is protected since 1999 (Presidential decree, No. 321-900).
- In India it is protected since 2001 by the Indian Ministry of Environment and Forests.
- In Mexico it has total protection (NOM-029-PESC-2000).
- In Maldives it is protected since 1995 by the Environment Law (4/93).
- In Philippines it has full protection since 1998 Administrative Order No. 193.
- In South Africa it has total protection.
- In Taiwan it is protected since 2002 by The Whale Shark Harvest Reporting System.
- In Thailand it is protected since 2000 through the Fishing Act B.E. 2490.

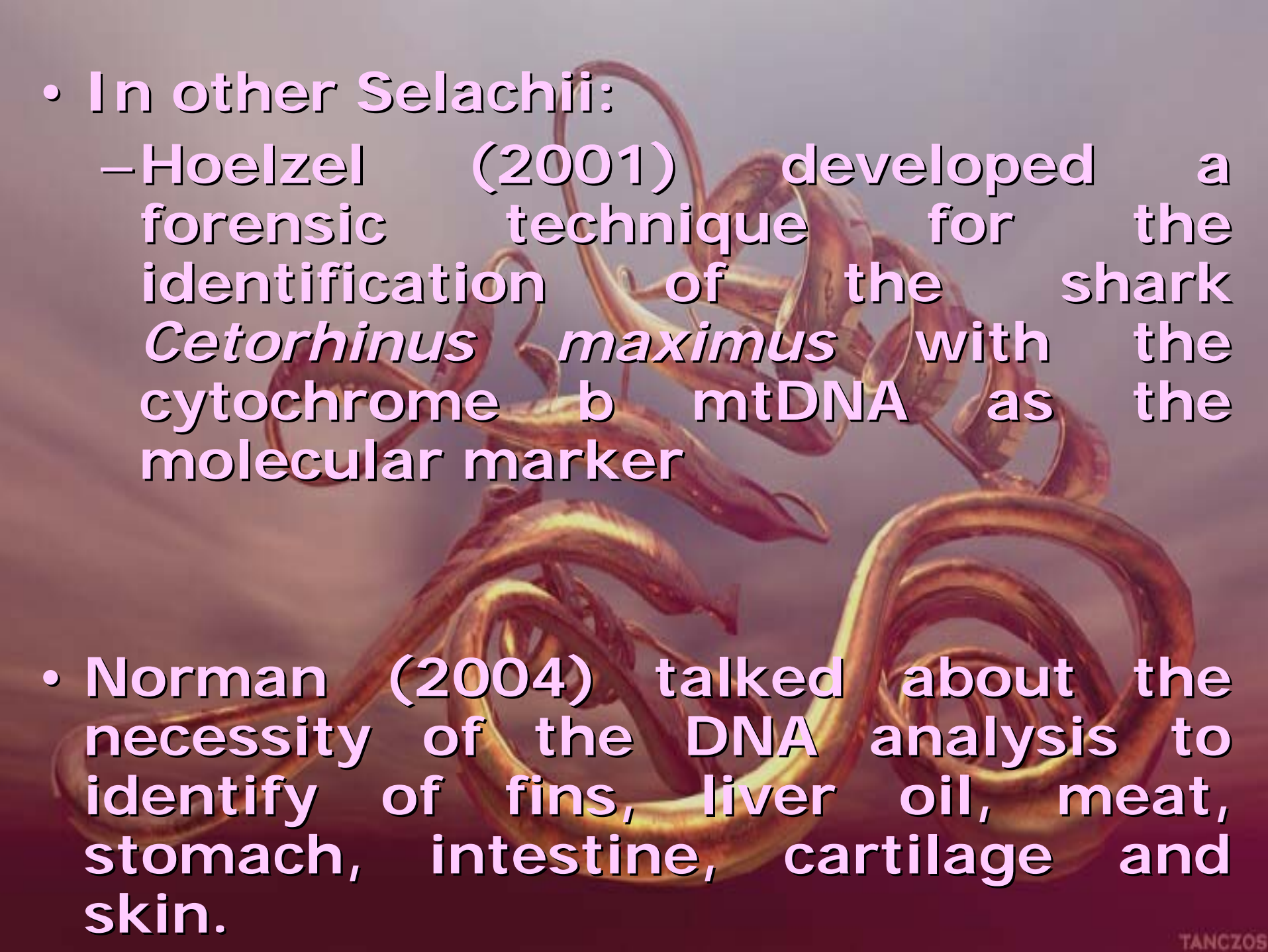


Joung et al. 1996

INTERNATIONAL PROTECTION

- The FAO in 1999 made an international action plan for the conservation and management of sharks.
- The WS is listed in the United Nations Fish Stocks Agreement and in the UN Convention of Law of the Sea.
- The International Union for the Conservation of Nature (IUCN) includes the WS as a vulnerable specie.
- The WS is listed in the CITES appendix number III and in the 12th Conference of Parties appendix number II in 2002.
- NGO participation in the WS conservation

- Genetic analysis is a very useful tool to support the conservation of species.
- The use of forensics applications and the expansion of the use of genetic tools for the acquirement of data from tissue examination has assisted the decision taking in the management of protected species.
- The present study pretends to implement a molecular technique capable of identifying any WS tissue.

- 
- In other Selachii:
 - Hoelzel (2001) developed a forensic technique for the identification of the shark *Cetorhinus maximus* with the cytochrome b mtDNA as the molecular marker
 - Norman (2004) talked about the necessity of the DNA analysis to identify of fins, liver oil, meat, stomach, intestine, cartilage and skin.

RATIONALE

- Because of its selected life history the WS is vulnerable to fisheries
- Furtive fishing persistence
- Forensic techniques are useful for the identification of species and means a support for the governments that are interested in combating furtive fishing.
- With a specific molecular marker of WS it is possible to distinguish WS products from others of different species

HYPOTHESIS

The amplification product of the control region hypervariable fragment mtDNA is specific for Whale Shark

OBJECTIVE

General objective

- To demonstrate the specificity of the primers which amplifies the control region hypervariable fragment mtDNA in WS

Particular objectives

- To Know the optimal conditions of this fragment amplification in WS
- To introduce an accessible and economical forensics method to identify WS products
- To reject a possible crossed amplification in other important commercial species

METHODOLOGY

Tissue obtention

Laboratory

DNA Isolation

Electrophoresis

The 18S Gene rDNA PCR, as first positive control
The PCR conditions has an initial denaturalization at 94°C for 3 min. Followed by 35 cycles of alternancy of T°:1 min. at 94°C, 1 min. at 57°C and 1 min. at 72°C.

Universal Primers PCR, in positive control

ThrFTB Forward – CTT GTA AGR CGA AGAS CTG GAG and 12 Srev 326 ACT CGT ATA ACC GCG GTG GCT (Sandoval-Castillo et al., 2004).

Universal primers annealing temperature optimization

Electrophoresis

Specificity test of the primers that amplify the mtDNA Control Region Hypervariable Fragment in WS

TB31F-5´ - TGCATGGTTTTATGTACGTC-3´ and RC-TB3R 5´ -GGCAGGTGTCGGAGCTT-3´ Ramirez- Macías *et al*, (2007).

The PCR conditions were: The initial denaturation was at 96°C for 3 min. Followed by 35 cycles of alternancy of T°: 1 min. at 94°C, 1 min. at 60.3°C and 1 min. at 72°C.

Cephaloscyllium ventriosum



Rhizoprionodon longurio

Carcharhinus falciformis



Carcharhinus limbatus

Carcharhinus leucas



Carcharhinus perezi



Prionace glauca



Mustelus henlei

Carcharodon carcharias



Isurus oxyrinchus

Sphyrna lewini



Sphyrna zygaena

Squatina californica



Ginglymostoma cirratum



Inclusion criteria

- Only Sharks were included
- It was necessary to be certain of species
- All the sharks DNA tested in the specificity probe should have passed the control region amplification with the universal primers.



RESULTS AND DISCUSSION

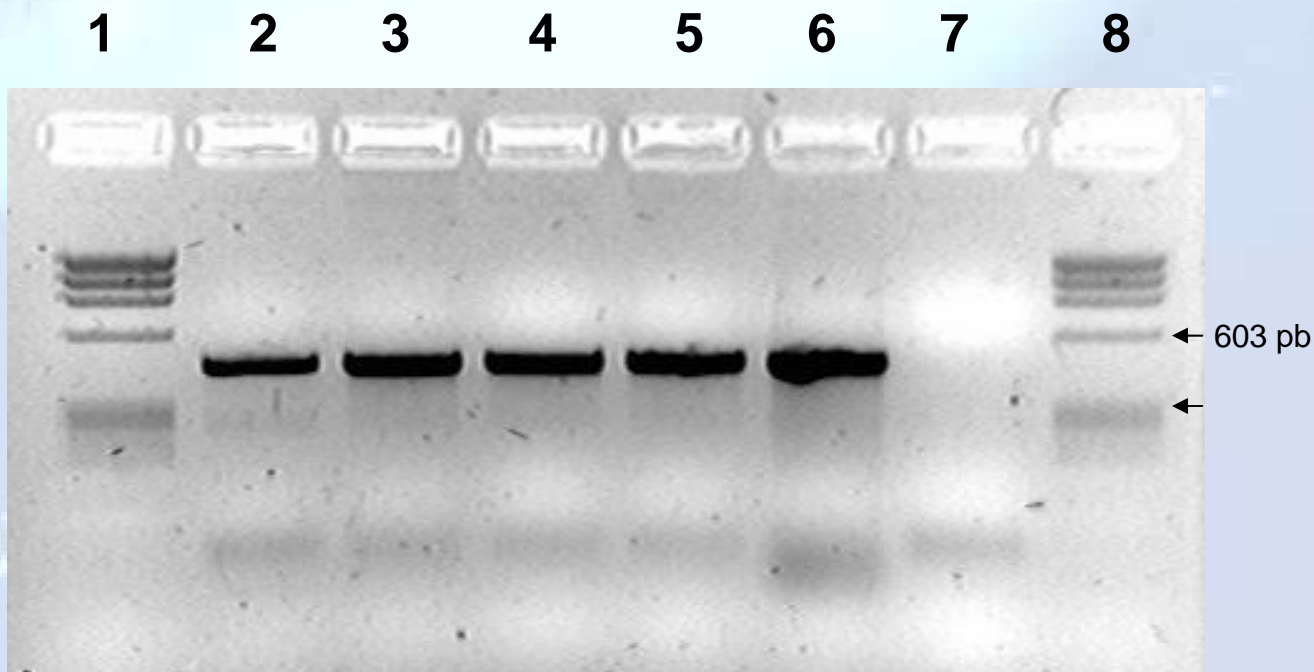
DNA Evaluation, 18S Gene Amplification in WS

LANES

1. MWM PHIX174
2. WS 1
3. WS 2
4. WS 3
5. WS 4
6. + CONTROL CUL123
7. - CONTROL
8. MWM PHIX174

PCR CONDITIONS

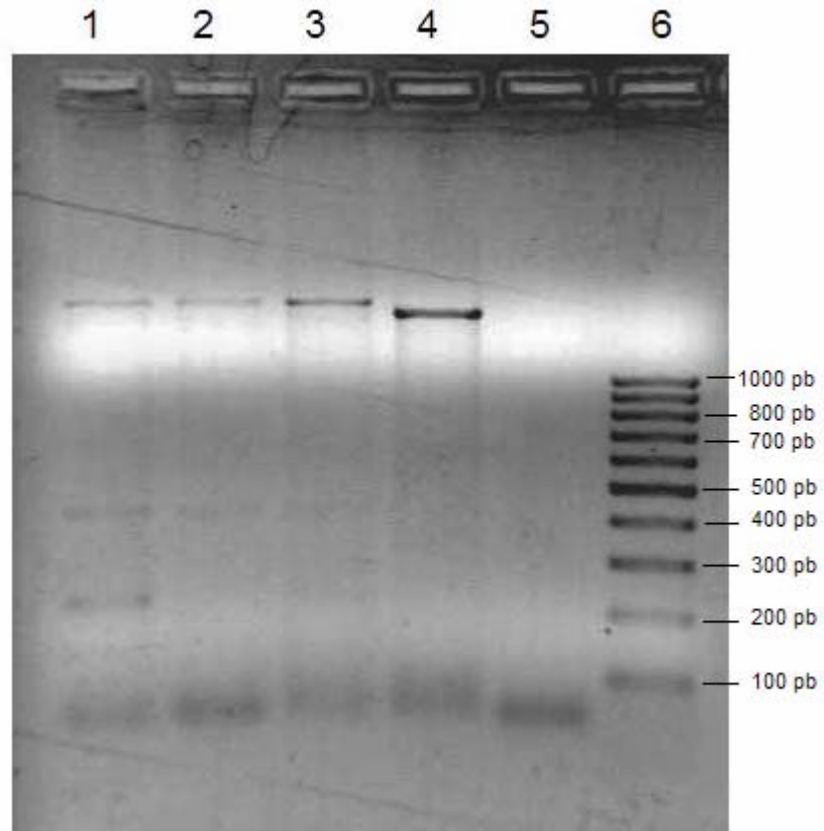
- | | | |
|-----|------|-------|
| 1X | 96°C | 3 MIN |
| 35X | 94°C | 1 MIN |
| | 57°C | 1 MIN |
| | 72°C | 1 MIN |



Control region mtDNA WS Amplification with the Universal Primers, in positive control

LANES

1. WS 2
2. WS 3
3. WS 4
4. + CONTROL
5. - CONTROL
6. MWM



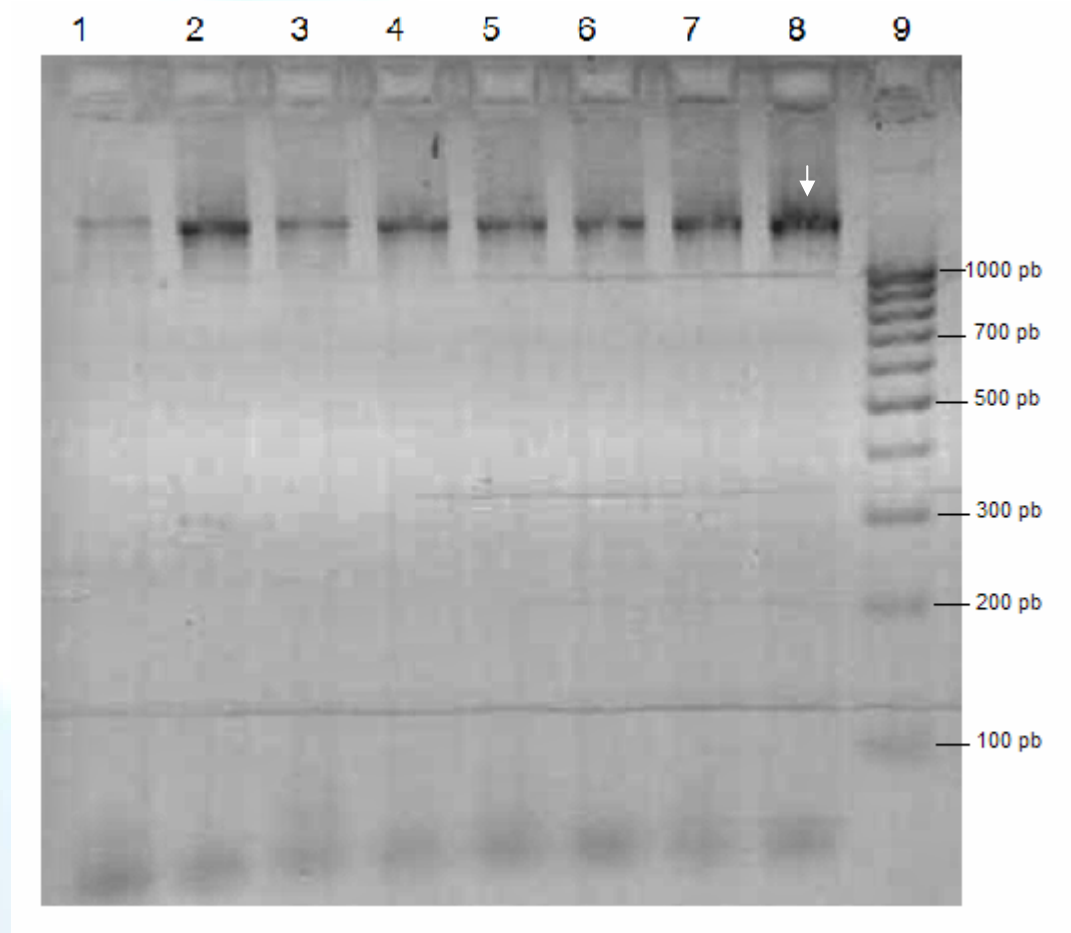
PCR conditions for the Universal Primers Standardization

LANES °C

1. 62
2. 61.5
3. 60.3
4. 58.4
5. 55.9
6. 54.1
7. 52.8
8. 52.0
9. MWM

PCR CONDITIONS

- 1X 96°C 3 MIN
35X 94°C 1 MIN
52°C 1 MIN
72°C 1.5 MIN



Orectolobiformes control region amplification, in positive control

LANES

1 MWM

3 *G. cirratum* 1



There was positive amplification with one individual of this order

Carcharhiniformes control region amplification, in positive control

Positive amplification were obtained in 10 species of this order.
Those species are included in 4 families

Right

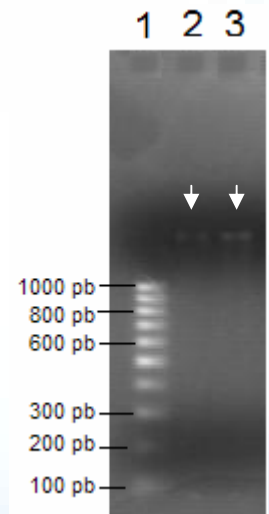
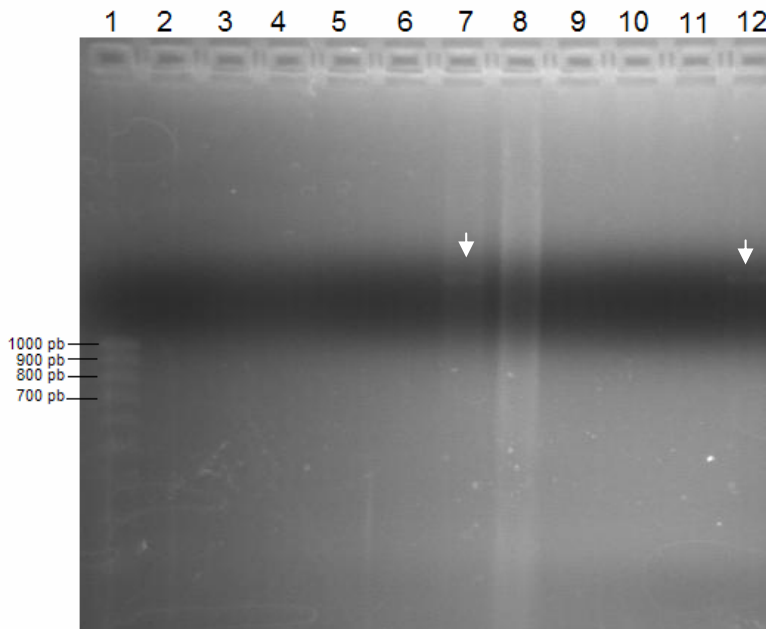
LANES

- 1 MWM 100 bp
- 7. *C. limbatus*
- 12. *M. henlei* 1

Left

LANES

- 1. MWM 100 bp
- 2. *M. henlei* 2
- 3. *M. henlei* 3

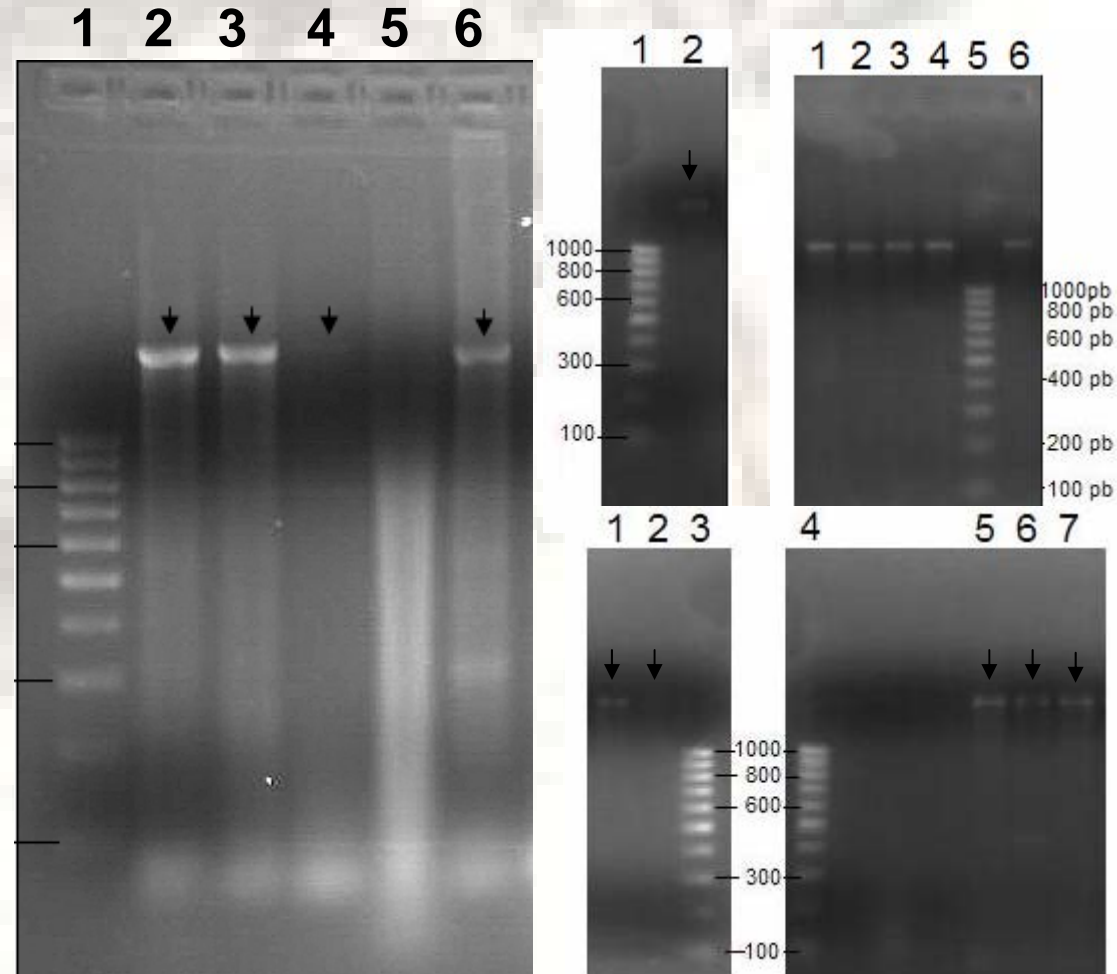


Carcharhiniformes control region amplification, in positive control

Right up	Down
1. <i>P. glauca</i> 1	
2. <i>P. glauca</i> 2	
3. <i>P. glauca</i> 3	
4. <i>C. perezi</i>	MWM
5. MWM	<i>S. zygaena</i> 1
6. <i>C. perezi</i> 2	<i>S. zygaena</i> 2
7.	<i>S. zygaena</i> 3

Middle up	
1. MWM	<i>S. lewini</i> 1
2. <i>C. falsiformis</i>	<i>S. lewini</i> 2
3.	MWM

Left
1. MWM
2. <i>C. leucas</i> 1
3. <i>C. leucas</i> 2
4. <i>R. longurio</i>
6. <i>C. ventriosum</i>



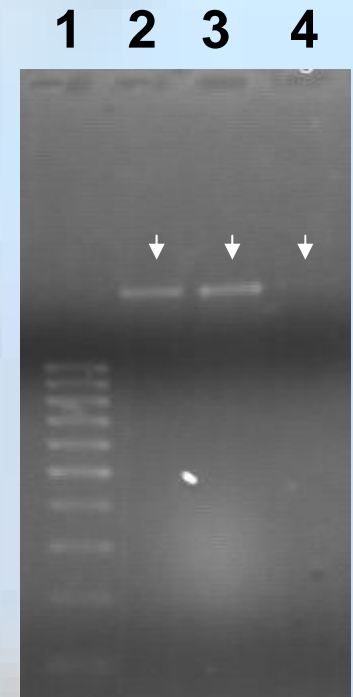
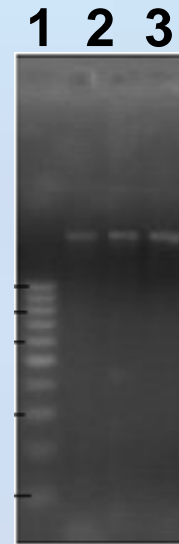
Lamniformes control region amplification, in positive control

Left

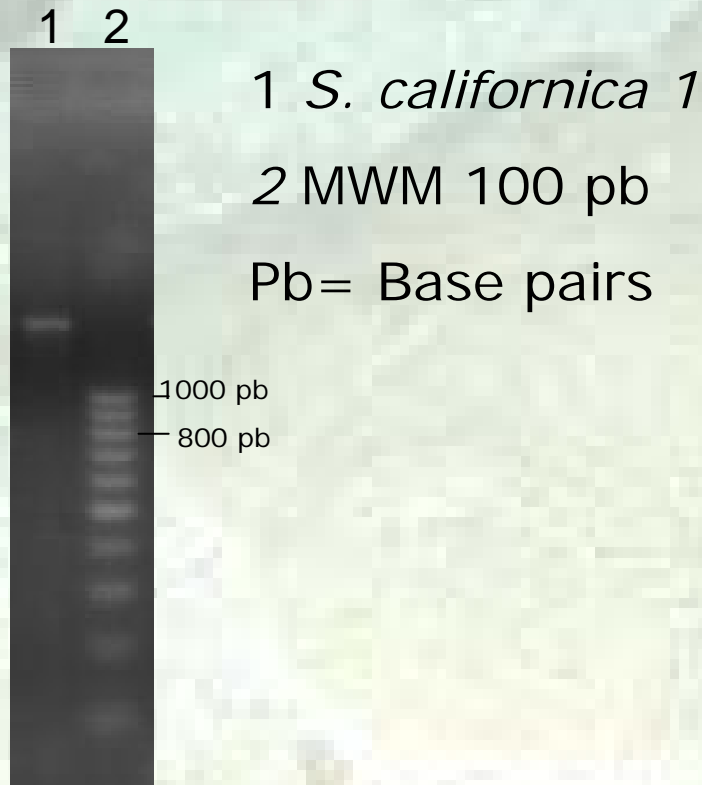
1. MWM 100 pb
2. *I. oxyrinchus* 1
3. *I. oxyrinchus* 2
4. *I. oxyrinchus* 3

Right

1. MWM 100pb
2. *C. charcharias* 1
3. *C. charcharias* 2
4. *C. charcharias* 3



Squatiniiformes control region amplification, in positive control

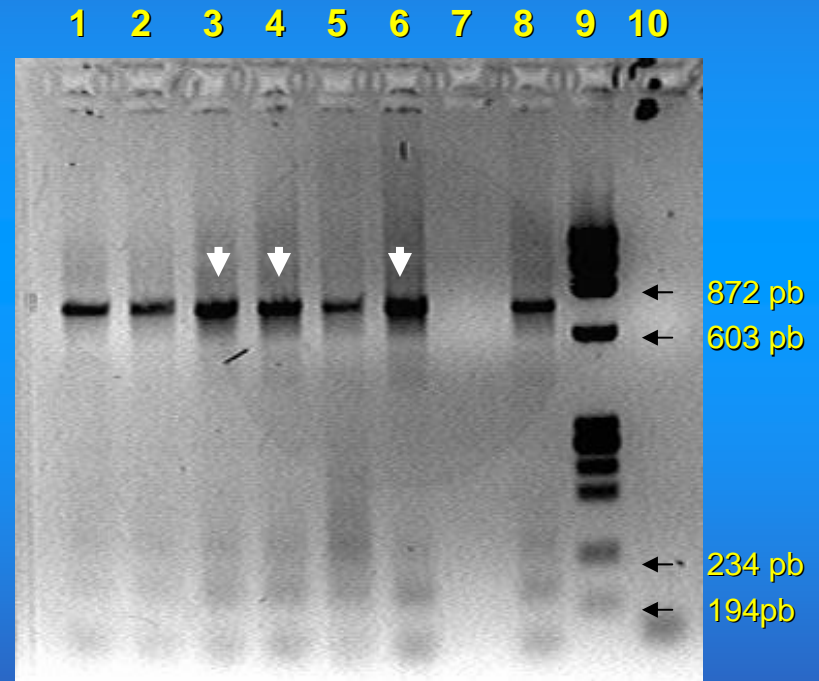


There was positive amplification with one individual of this order.

GRADIENT PCR SPECIFIC PRIMERS AMPLIFICATION

LANES °C

1. 62
2. 61.5
3. 60.3
4. 58.4
5. 55.9
- 6. 54.1**
7. 52.8
8. 52.0
9. PHIX174
10. NEGATIVO



The implemented PCR conditions were similar to those 18S gene amplification, except by the annealing temperature, which was made in gradient.

Primers that amplify the Control Region Hypervariable Fragment in WS Specificity test

PCR CONDITIONS

1X 96°C 3 MIN
35X 94°C 1 MIN
54.1°C 1 MIN
72°C 1 MIN

LANES

1 MPM
2 WS
3 *C. falsiformis*
4 *P. glauca* 1
5 *P. glauca* 2
6 *P. glauca* 3
7 *M. henlei* 4

LANES

8 *M. henlei* 2
9 *M. henlei* 3
10 *S. lewini* 1
11 *S. lewini* 2
12 *S. zygaena* 1
13 *S. zygaena* 2
14 *S. zygaena* 3

1 2 3 4 5 6 7 8 9 10 11 12 13 14



Primers that amplify the Control Region Hypervariable Fragment in WS Specificity test

PCR CONDITIONS

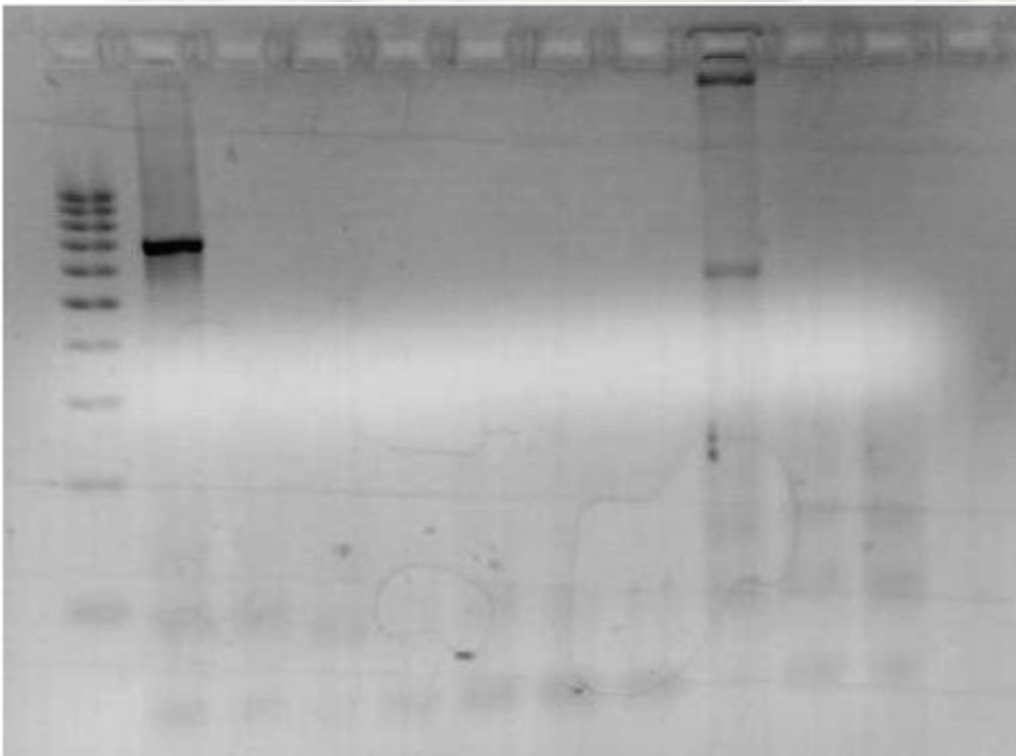
1X 96°C 3 MIN

35X 94°C 1 MIN

54.1°C 1 MIN

72°C 1 MIN

1 2 3 4 5 6 7 8 9 10 11



LANES

1 MWM

2 WS

3 *C. charcharias* 1

4 *C. charcharias* 2

5 *I. oxyrinchus* 1

6 *I. oxyrinchus* 2

7 *I. oxyrinchus* 3

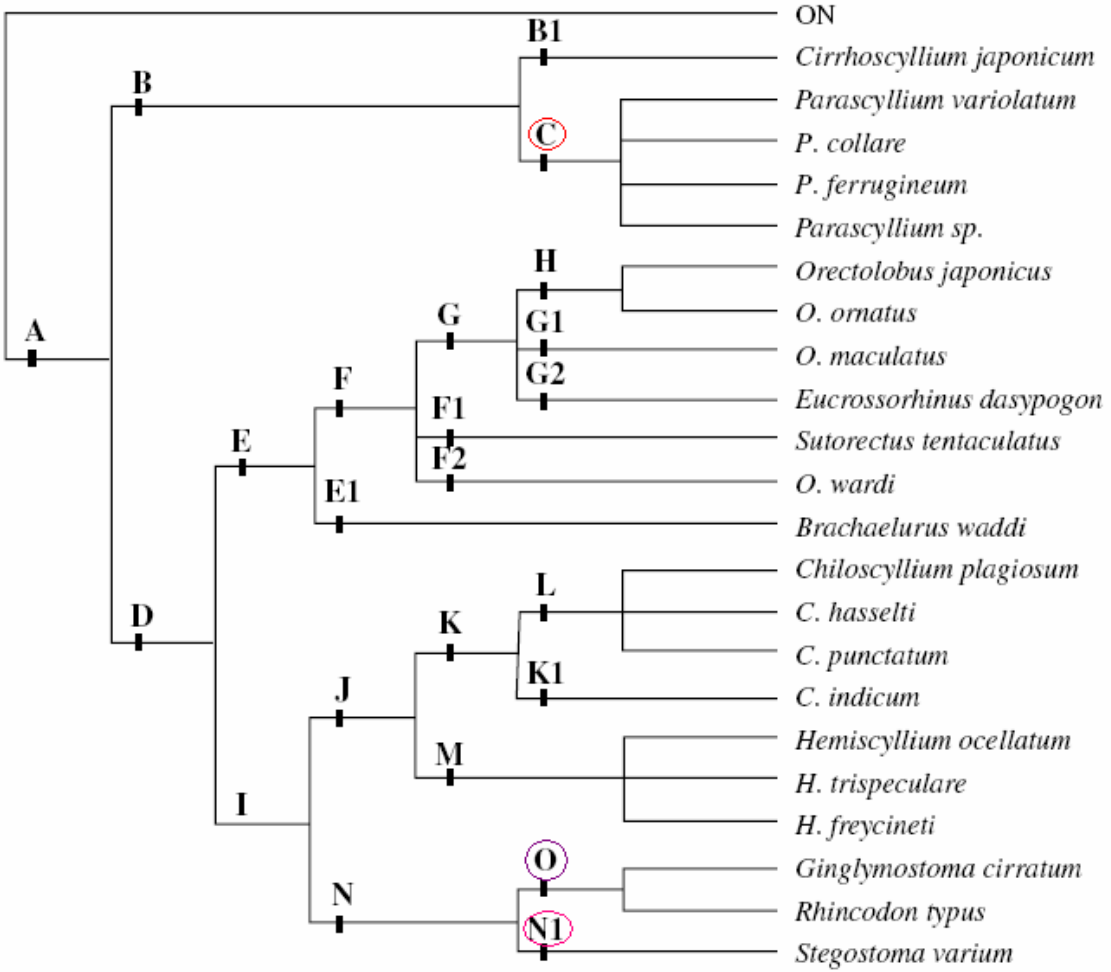
8 *S. californica* 1

9 ***G. cirratum*** 1

10 *C. perezii* 1

11 *C. perezii* 2

Genetics nearness and crossed amplification



Parascyllidae (Gill, 1862)

Brachaeluridae (Applegate, 1972)



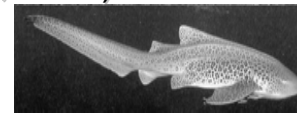
Orectoloboidae (Jordan y Fowler, 1903)



Hemicylliidae (Gill, 1862)



Ginglymostomatidae (Gill, 1862).



Stegostomatidae



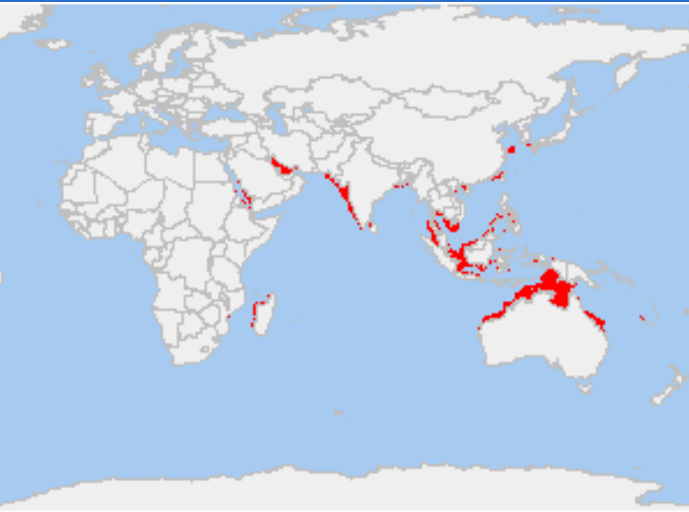
Rhincodontidae (Smith, 1929)

Goto, T., 2001. *Mem. Grad. Sch. Fish. Sci. Hokkaido Univ.* 48(1):1-100.

Compagno, 1977



Genetics nearness to the WS. Distribution and commercial importance

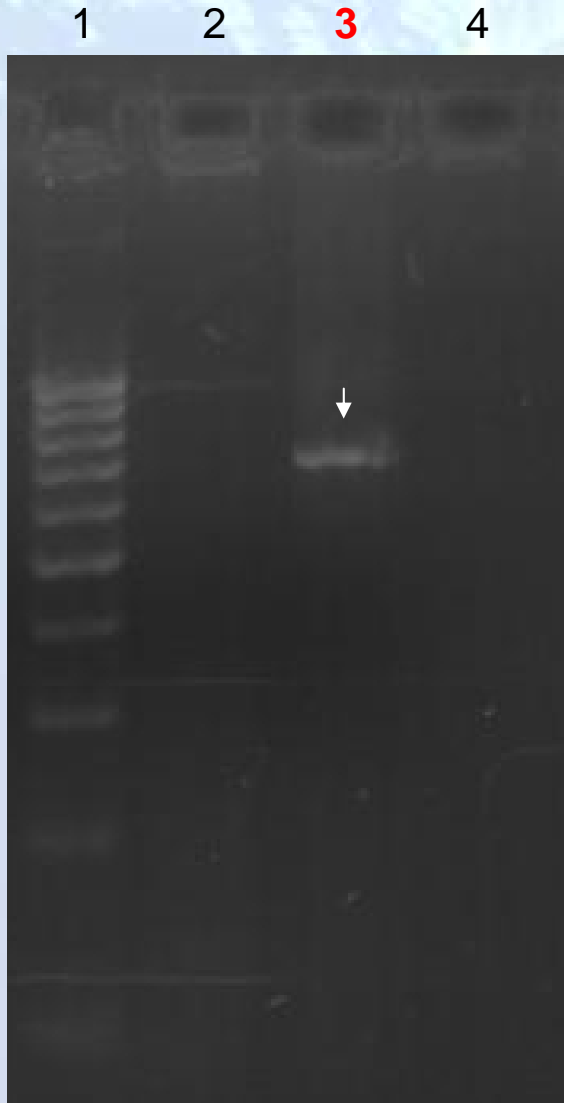


Stegostoma is taken in Pakistan, India, Thailand, Malaysia, Taiwan (Province of China), and elsewhere where it occurs

(www.fao.org)



Specificity PCR conditions of the primers that amplify the Control Region Hypervariable Fragment in WS



PCR CONDITIONS

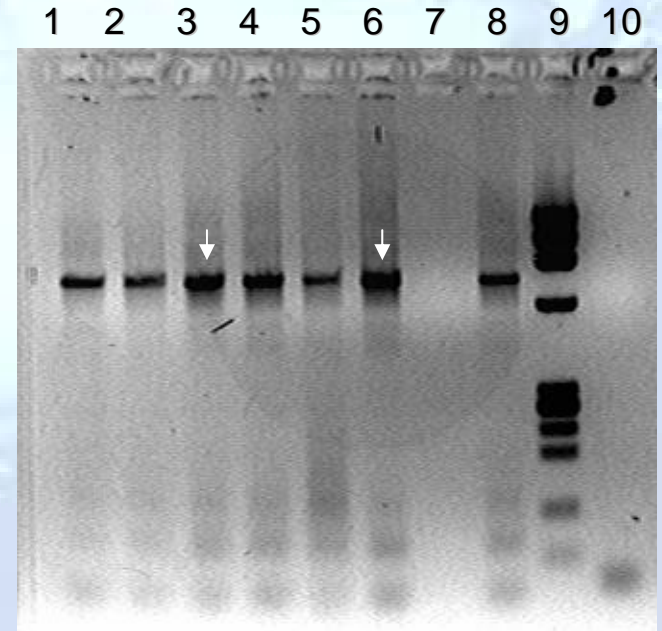
1X 96°C 3 MIN
35X 94°C 1 MIN
60.3°C 1 MIN
72°C 1 MIN

1 MWM 100bp

2 *G. cirratum* 1

3 WS

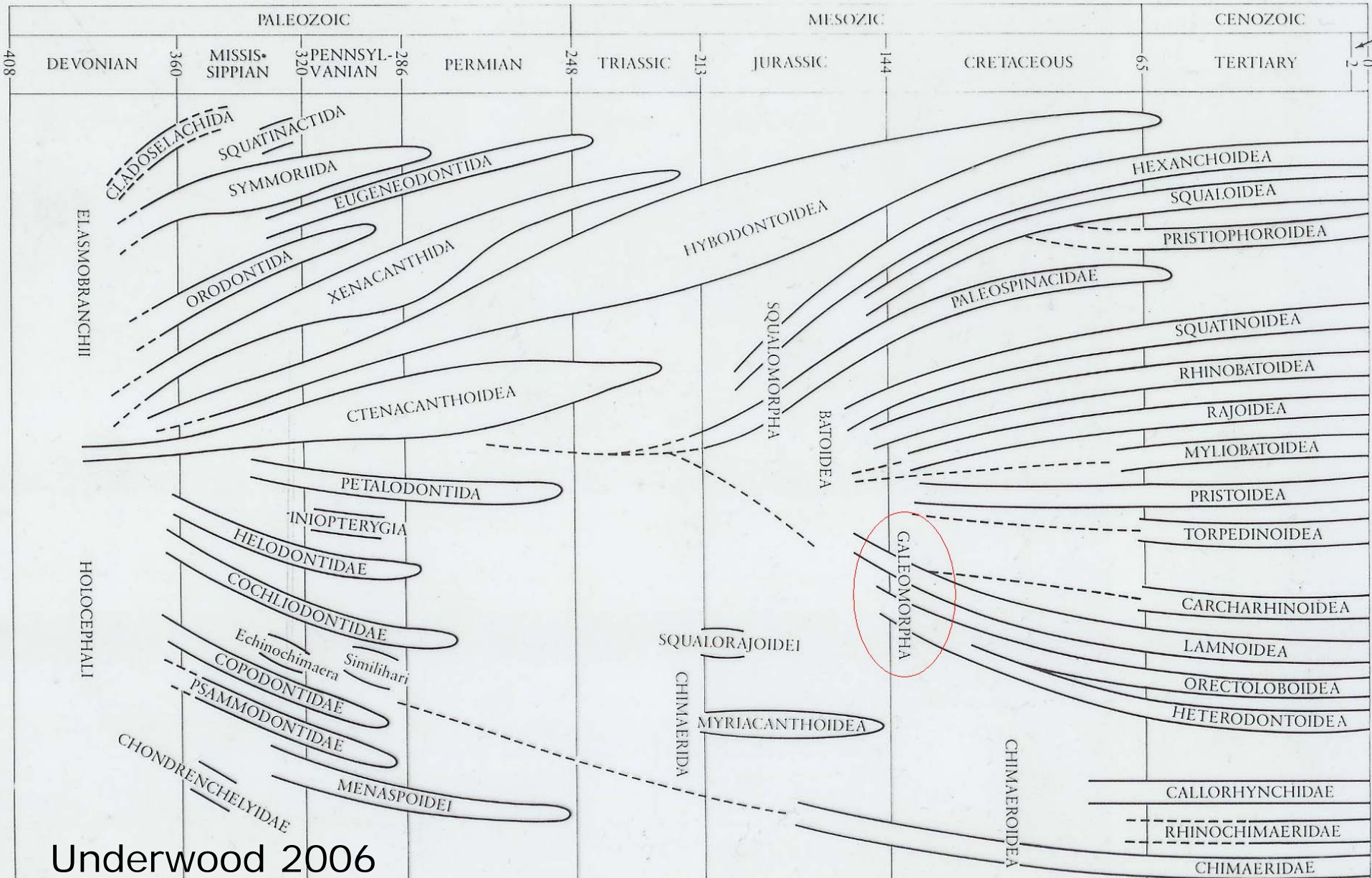
4 Negativ



LANES °C

- 62
- 61.5
- **60.3**
- 58.4
- 55.9
- 54.1
- 52.8
- 52.0
- PHIX174

Genetics nearness and crossed amplification



CONCLUSIONS

- The primers that amplified the control region hypervariable fragment in WS are specific for the WS under the following conditions: initial denaturation at 96°C for 3 min. Followed by 35 cycles of alternancy of T°:1 min. at 94°C, 1 min. at 60.3°C and 1 min. at 72°C.
- Nonetheless, the primers should be tested with the most closely related species of commercial importance.
- The control region of mtDNA may be used as a specific molecular marker of WS. Also, it could mean a support for the governments that are interested in combating furtive fishing of WS for its conservation.

References

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- Underwood. 2006. Diversification of the Neoselachii (Chondrichthyes) during the Jurassic and Cretaceous. *Paleobiology*, 32(2) pp. 215–235

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