Final Report

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Project Title: Discrimination of Cownose Ray, *Rhinoptera bonasus*, Stocks Based on Microsatellite DNA Markers

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Objectives

The objective of this project was to develop and use microsatellite DNA markers to discriminate stocks of the cownose ray, *Rhinoptera bonasus*. The specific objectives that were approved in the grant application were to:

- Collect neonate and/or pregnant female cownose rays from the Chesapeake Bay and the Gulf of Mexico.
- Create a suite of molecular markers specific to cownose rays.
- Screen these new markers for reliability and variability in the cownose ray
- Analyze cownose ray samples collected from the nursery grounds in the Chesapeake Bay and Gulf of Mexico to look for evidence of stock structure and to get baseline estimates of genetic diversity.

All of the objectives set forth in the original grant were accomplished.

Methods

Sample Collection

Samples were collected from pound net operations in Chesapeake Bay as well as from Pamlico Sound, NC and Sandy Hook Bay and Cape May, NJ. In the eastern Gulf of Mexico, samples were taken from Tampa Bay and Crooked Bay, FL. Total disc length, sex, and maturity state were recorded. A small tissue sample was taken from the trailing edge of the pectoral fin and placed in 95% ethanol until DNA isolation. When pregnant females were sampled, the pup was removed, measured and a tissue sample was taken (Table 1). However, no mother pup pairs were analyzed to avoid biasing results due to relatedness.

Marker development

High molecular weight genomic DNA was isolated from tissue taken from a cownose ray caught in Chesapeake Bay Virginia within 12 hours of capture and sent to the Virginia Bioinformatics Institute at Virginia Tech for sequencing on an Ion Torrent personal genome machine (PGM, Life Technologies, Carlsbad, CA). After sequencing low quality sequencing reads were filtered (removed) using the GALAXY software package (Goeks et al. 2010, Blankenberg et a1. 2010). After filtering, sequences were assembled into contigs using the CLC GENOMICS WORKBENCH software (CLC Bio, Aarhus, Denmark) and the resulting contigs were searched for sequences containing microsatellites using the MSATCOMMANDER software package (Faircloth, 2008). Primers were designed using PRIMER3 (Rozen and Skaletsky 2000).

Marker optimization and statistics

Once designed, primers were used to amplify targeted loci using standard PCR protocols, and the resulting amplification products were electrophoresed against a 1 Kb + size standard (Invitrogen, Grand Island, NY) on submerged horizontal agarose gels to assure that products amplified successfully and were of the expected size. Markers found to reliably amplify a product of the expected size were evaluated for a subset of Chesapeake Bay and the eastern Gulf of Mexico samples (n = 24 each) to assess amplification consistency and levels of polymorphism as follows. DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. PCR reactions were carried using Qiagen (Valencia, CA) reagents and fluorescently labeled primers. The resulting PCR products were separated on an ABI 3130*xl* Prism Genetic Analyzer (Applied Biosystems, Foster City, CA) with a GeneScan 500-LIZ size standard (Applied Biosystems, Foster City, CA). The chromatic peaks for each microsatellite locus were scored using the GENEMARKER AFLP/Genotyping Software, v1.75 (SoftGenetics, State College, PA). The GENEPOP'007 software package was used to test for deviations of genotypic distributions from HWE expectations (exact tests, Guo and Thompson 1992) and the ARLEQUIN v 3.5 software package (Excoffier and Lischer 2010) was used to estimate Weir and Cockerhams' (1984) unbiased estimator of Wrights *F*-statistics. Significance was assessed via permutations of the data. To assess the presence of hierarchical genetic structure, an AMOVA was conducted in ARLEQUIN v 3.5 among alternate groupings of sample collections to maximize the amount of variance due to variation among groups of collections. A multidimensional factorial correspondence analysis was used to visualize the relationships among individuals with no a priori expectation of group membership using each allele as an independent variable in GENETIX ver. 4.05 (Belkhir et al. 1996-2004). In addition, a principal components analysis based on the gene frequency data was conducted in PCAGEN (available at: http://www2.unil.ch/popgen/softwares/pcagen.htm).

Mitochondrial DNA

Although it was not originally a goal of the grant, due to the fact that a subset of samples from the eastern Gulf of Mexico either did not amplify well with the microsatellite markers being developed for this study or had alleles that were very different from those found in other samples across several loci, primers were designed for several mitochondrial (mtDNA) loci (cytochrome oxidase 1 (COI), ctyochrome *b* (cyt*b*), 12S ribosomal RNA (12S), and NADH Dehydrogenase Subunit 2 (ND2), subunit 4 (ND4) and subunit 5 (ND5)) based on alignment of sequences available for other Myliobatidae available in GenBank. Preliminary analysis of 8 samples suggested that primers designed to amplify an 850 bp region of the ND2 locus exhibited the most variability and thus ND2 was used for amplification and sequencing of subsequent samples (RHIN_ND2_F1: GAACCCYTTAATCCTCTYCATC; RHIN_ND2_R3: ATRGGGGTTAATGGRAGRAG). Qiagen reagents were used to amplify template DNA via polymerase chain reaction (PCR). Amplification products were cleaned using the Q1Aquick PCR Purification Kit (Qiagen) and sequenced using the BigDye® Terminator ver. 3.0 Cycle Sequencing Kit (Applied Biosystems®, Foster City, CA, USA) at a 1:8 dilution. Sequenced samples were separated on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems®), and edited using the SEQUENCHER 4.8 (Gene Codes Corp., Ann Arbor, MI, USA) software package, aligned using one of the MAFFT algorithm (Katoh et al. 2005) available in GENEIOUS PRO 5.4.6 (Biomatters, available from http://www.geneious.com/) and the relationships among samples were visualized using a neighbor-joining or UPGMA algorithm.

Nuclear RAG-1 locus

Since mitochondrial DNA is clonally inherited, it is difficult to discriminate signatures of historical events from contemporary processes. Therefore, to further elucidate the patterns found among samples, primers were designed for the nuclear recombination activating gene 1 (RAG1)

based on sequences from other Myliobatidae available in GenBank (Rhin_5'RAG1_F: CATCCCACCCACTTCTGC; Rhin_5'RAG1_R: TCAGAAACGTACTAATCCTAATGGC). The resulting amplification products were cloned using a TA Cloning Kit (Invitrogen), 3-5 clones were selected from each sample and sequenced, aligned, and relationships among sequences were visualized as for mtDNA above.

Results

Ion Torrent sequencing of genomic DNA extracted from cownose ray tissue resulted in the generation of 639,000 reads ranging from 5 – 398 base pairs (bp) in length (average 175 bp) and an average sequence quality of 30. After filtering out low quality sequences, defined as sequences with a quality score lower than Q10, 16% of sequences (100,585) were discarded, leaving 526,200 sequences of sufficient quality for further analysis. After the remaining high quality sequencing reads were assembled into contigs, the contigs were searched for those containing perfect di- tri- or tetra-nucleotide repeats. Approximately 1,767 microsatellite containing sequences were found and primers were designed for 259 of these sequences as they were found to have sufficient flanking sequence for primer design. Primer pairs for 96 microsatellite ordered for testing and optimization (Table 2).

Of the 96 primer-pairs ordered, 12 (Rbon_1, Rbon_30, Rbon_37, Rbon_38, Rbon_41, Rbon_52, Rbon_56, Rbon_69, Rbon_75, Rbon_78, Rbon_79 and Rbon_80) were successfully optimized. These markers were found to amplify consistently and alleles could be clearly discriminated. In addition, all were found to be in conformance to the expectations of Hardy-Weinburg equilibrium in the subset of samples tested. This panel of microsatellite markers was used to generate multilocus genotypes for 88 samples from Chesapeake Bay off the coast of Virginia (VA), 8 samples from Pamlico Sound, North Carolina (NC), 33 samples from Sandy Hook Bay and Cape May, New Jersey (NJ). In total, 129 samples were analysed from the U.S. east coast. In addition, 102 samples from the eastern Gulf of Mexico off the coast of Florida were analysed, 42 from the area around Crooked Bay (CB) and 68 from Tampa Bay (TB).

During optimization of microsatellite loci, it became apparent that there were samples that either would not amplify or that had alleles that were very different in size from the rest of the test samples. This caused some concern that misidentifications had occurred in the field and tissue samples had been taken from the wrong species. However, as *Rhinoptera bonasus* is the only rhinopterid reported to occur along the U.S. east coast and Gulf of Mexico, the results were perplexing. To examine the possibility of misidentification, an 850 bp region of the mitochondrial ND2 gene region was amplified from 207 of the samples and sequenced. These sequences fell into three distinct groups (clades), two of which were found in both the Chesapeake Bay and Gulf of Mexico and one that was only found in the Gulf of Mexico (Figure 1). Overall, ND2 sequences were found that differed at 48 of 753 base pairs (6.4%) between groups, which is on the order of differences generally seen between species. In order to assess whether the differences between the groups of sequences were due to long term historical isolation (vicariance) or were reflective of actual boundaries between species, 800 bp of the nuclear RAG1 locus were amplified, cloned and sequenced from 5-10 individuals from each of the three groups (5 clones/individual) to look for concordance between marker classes. Comparison of the results of both data sets suggests that both of the mitochondrial clades that are distributed throughout the Gulf of Mexico and Chesapeake Bay, although they are distinct based on ND2 sequences, are indistinguishable based on RAG1 sequences. This suggests that the two mitochondrial groups resulted from a historical boundary that has since been removed (Figure 2). Interestingly, the third mitochondrial group was found to have RAG1 sequences that clearly corroborate the separation based on ND2 (Figure 2). This suggests the possibility of a cryptic species in the Gulf of Mexico.

To explore the possibility of a second, heretofore undocumented species in the Gulf of Mexico, we contacted international colleagues and collected samples from other geographic locations.

Although this was not part of the original grant, we felt that it was imperative to try to identify whether this was a second species since this would greatly affect the interpretation of the results. To this end, we received six tissue samples from Brazil, four of which were identified as Ticon (Brazilian) cownose ray, *R. brasiliensis* which is considered to be an endemic species limited to an 1800 km stretch of coastline off the coast of Brazil between Rio de Janeiro and Rio Grande do Sul (Bigelow and Schroeder 1953, Menni and Stehmann 2000). *Rhinoptera brasilensis* and two identified as *R. bonasus*. In addition we received three samples identified as *R. bonasus* from Manaura, La Gauijra Columbia in Caribbean Sea. We also attempted to get samples of the Lusitanian cownose ray, *R. marginata*, known from the western coast of Africa and Mediterranean Sea, however we were not successful. DNA was isolated from these samples as above and ND2 sequences were generated. The results of the molecular sequence data suggest that the other species bears a close genetic affinity to the Ticon (Brazilian) cownose ray, *R. brasiliensis*. Samples from the ND2 clade found to be present only in the Gulf of Mexico had sequences very similar and in some cases identical to samples identified as *R. brasiliensis* (Figure 3). Samples found to belong to this group based on mtDNA sequencing will hereafter be referred to as *R.* aff. *brasiliensis*. In addition, the molecular data indicate that the *R. bonasus* samples collected from Columbia and Brazil formed a distinct clade that was very close to but distinct from *R. bonasus* samples taken from the Gulf of Mexico and Chesapeake Bay, suggesting that the Caribbean Sea may harbor a separate genetic stock and that there may be a nursery area in the Caribbean Sea. All of these findings point to the need for more work on the stock structure of *R. bonasus*, as it is clearly more complex that previously thought. Once we were able to confidently identify *R.* aff. *brasiliensis*, these samples were separated from samples identified as R. bonasus. In Tampa Bay 5 of 68 samples (17%) were identified as *R.* aff. *brasiliensis* while 18 of 42 (43%) of samples taken from Crooked Island Sound were identified as *R. aff. brasiliensis*.

All samples were amplified with 12 of the microsatellite loci developed for this study. These included Rbon_1, Rbon_30, Rbon_37, Rbon_38, Rbon_41, Rbon_52, Rbon_56, Rbon_69, Rbon_75, Rbon_78, Rbon_79 and Rbon_80. Multilocus genotypes were generated for all *R. bonasus* samples including samples taken from Columbia and Brazil. Multilocus genotypes were also generated for all samples identified as *R.* aff. *brasiliensis*, however, three of the loci; Rbon_30, 79 and 80 failed to amplify well for these samples and were excluded from comparisons among putative species. Both a factorial correspondence analysis and a principal component analysis based on microsatellite loci corroborate the results based on sequencing of the mitochondrial ND2 and nuclear RAG1gene regions, suggesting the presence of three distinct groups: R bonasus collected from the Gulf of Mexico and Chesapeake Bay, R. bonasus collected from Brazil and Columbia and *R.* aff. *brasiliensis* collected from the Gulf of Mexico plus *R. brasiliensis* collected from Brazil (Figure 4).

Finally, multilocus microsatellite genotypes from samples identified as *R. bonasus* from the Gulf of Mexico and Chesapeake Bay were analyzed for conformance to the expectations of Hardy-Weinburg equilibrium (HWE). All loci were in HWE with the exception of Rbon_52, which showed evidence of a heterozygote deficiency, suggesting the possibility of a null allele. All analyses were subsequently conducted both with and without this locus. The number of alleles/locus ranged from three at Rbon_80 and Rbon_78 to 12 at Rbon_79.

Pairwise F_{ST} values between samples taken from CB and TB in the Gulf of Mexico were not significantly different (F_{ST} = 0.0067, P= 0.152+/-0.003). Similarly, there were no significant differences among samples taken from the U.S. east coast (NC, VA, NJ). F_{ST} values ranged from -0.0036 between VA and NJ (P = 0.513+/- 0.019) to 0.0035 between NC and NJ (P= 0.332 +/- 0.018). An AMOVA, which grouped GOM samples together and U.S. east coast samples together found that a significant component of the variance was due to variation among groups F_{CT} 0.02462, P< 0.0001 indicating that the distribution of allele frequencies differed significantly

between the Gulf of Mexico and U.S. east coast and suggesting that they comprise independent stocks.

Conclusions

All of the goals of the goals outlined in the original grant were met. Specifically, pregnant and neonate cownose rays were collected from the Chesapeake Bay and the Gulf of Mexico. The original grant stated that a suite of 8-10 microsatellite loci would be developed and characterized. This study resulted in isolation of over 200 potential loci and 12 loci were well characterized. Primer pairs are available for the remaining loci and will be made available in the form of a peer-reviewed publication. In addition, primer sequences for all 95 primers tested in this study are included in this report (Table 2). The subset of 12 well-characterized markers developed during this study were used to assess the presence of stock structure among cownose ray samples taken from nursery areas in the Gulf of Mexico and Chesapeake Bay. The resulting multilocus genotypes were analyzed for evidence that cownose rays in the Gulf of Mexico and Chesapeake Bay (U.S. east coast) are comprised of distinct stocks. We found significant genetic differences between the two major sampling areas based on our microsatellite data suggesting the presence of at least two stocks of *Rhinoptera bonasus*.

This grant was particularly difficult and resulted in more work than was expected or budgeted. Specifically, it became apparent that there were two distinct groups of *Rhinoptera* present in the Gulf of Mexico and this made optimization of microsatellite loci very difficult until we were able to use alternative genetic markers (mitochondrial ND2 and nuclear RAG1 sequences) to verify and separate the groups. This has resulted in preliminary data based on ND2, RAG1 and microsatellite loci suggesting that, rather than a single species, there are possibly two species of cownose ray in the eastern Gulf of Mexico. One of these species is *R. bonasus*, the species commonly described as being present throughout the western Atlantic from southern Massachusetts to Florida, the Gulf of Mexico and southern Brazil (Blaylock 1993, Neer and Thompson 2005). Based on molecular data, the other species bears a close genetic affinity to the Ticon (Brazilian) cownose ray, *R. brasiliensis* which is considered to be endemic off the coast of Brazil between Rio de Janeiro and Rio Grande do Sul (Bigelow and Schroeder 1953, Menni and Stehmann 2000). It is not known whether the presence of this species in the Gulf of Mexico represents a recent range expansion, a case of taxonomic confusion due to the lack of morphological characters to distinguish these species, a new species that is closely related to *R. brasiliensis*, or is another Atlantic rhinopterid that was not examined in this study, such as *R. marginata*. Interestingly, based on our data, this species comprises approximately 21% of our eastern Gulf samples, suggesting it is makes up significant proportion of *Rhinoptera* in the Gulf of Mexico. We have not seen evidence of *R.* aff. *Brasiliensis* off the coast of Chesapeake Bay, Virginia based on limited sampling effort (N=98).

In addition, genetic analysis of a limited number of tissue samples from cownose rays captured off the Caribbean coast of Columbia and from the coast of Brazil form a clade that is closely related to but divergent from *R. bonasus* samples taken from the Gulf of Mexico and Chesapeake Bay, suggesting that the Caribbean Sea may harbor a separate genetic stock. Our preliminary results are based on a relatively limited data set and we do not have voucher specimens that will allow us to correlate morphological and meristic characters with genetically distinct lineages. Also, we did not have the resources to obtain samples of the Lusitanian cownose ray, *R. marginata*, which occurs in the Mediterranean Sea and off the coast of West Africa.

In summary, it is apparent from the results of this study that 1) *R. bonasus* from the Gulf of Mexico and U.S. east coast are comprised of distinct genetic stocks based on microsatellite data 2) A limited number of samples of *R. bonasus* taken from Columbia and Brazil suggest that these samples comprise a third stock, distinct from the Gulf of Mexico and Chesapeake Bay stocks. 3) The number of stocks of cownsose rays that are present throughout their range is unknown and should be studied further. 4) There is potentially a second species of *Rhinoptera* that may make

up a significant portion of the cownose rays inhabiting the Gulf of Mexico. However, no samples were taken from the western Gulf of Mexico for this study, so the extent of the range in the Gulf of Mexico is unknown. This species bears a close genetic affinity to *R. brasiliensis* based on ND2, RAG1 and microsatellite markers. Whether this represents and extension of the previously known range of *R. brasiliensis* or whether it is a previously undescribed species warrants further examination.

This program will continue in the future as follows. We plan to publish the results of this study in a peer-reviewed journal. We also pan to continue to collect samples as they become available and plan to apply for funding in the future so we can continue to elucidate the patterns of stick structure in Atlantic rhinopterids.

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Table 1. Sample collections.

SAMPLE DATE	LOCATION	SAMPLES	ADULT MALES	ADULT FEMALES	MOTHERS WITH PUPS	JUVENILE MALES	JUNVENILE FEMALES
UNKNOWN	VA	8	0	3	3*	0	0
Aug. 2008	Lynnhaven,VA	25	0	0	0	16	9
May. 2009	Poquoson, VA	9	0	3	3*	0	0
Sept, 2010	Pamilco Sound, NC	4	UNKNOWN	UNKNOWN	UNKNOWN	UNKNOWN	UNKNOWN
Jun-Aug 2010	Crooked Island Sound,FL	11	2	2	0	5	2
2009-2010	Crooked Island Sound,FL	29	12	1	0	12	4
May-June 2009	VA	23	0	10	10*	0	0
June 2009	VA	18	2	8	8	0	0
June-July 2009	VA	13	3	5	5	0	0
June 2009	VA	11	1	6	4	0	0
Jun/July 2009	Eastern Shore, VA	5	2	3	0	0	0
May. 2013	VA	5	0	2	2*	0	0
Sept. 2011	Poquoson, VA	5	0	3	2	0	0
2010-2011	GOM, FL and VA	96**	9	9	0	43	33
May. 2012	Lynnhaven & Hampton (Amory), VA	43	7	20	11*	4	0
May, 2012	Hampton (Amory), VA	42	12	16	12	2	0
June, 2012	Lower Machodoc Creek, VA	6	1	3	2	0	0
June. 2012	Poquoson, VA	31	0	28	3	0	0
Aug. 2012	Brazil	6	UNKNOWN	UNKNOWN	UNKNOWN	UNKNOWN	UNKNOWN
?	Manaura, La Gauijra Columbia	5	UNKNOWN	UNKNOWN	UNKNOWN	UNKNOWN	UNKNOWN
Sept. 2012	Hampton,VA	36	0	29	3	3	1
Sept. 2012	Hampton,VA	10	0	5	2	0	3
Aug. 2012	Hampton,VA	5	0	0	0	0	5
Aug. 2012	VA	19	0	5	0	1	13
Sept. 2012	VA	1	0	1	1	0	0
2012	Cape May, NJ	21	4	0	0	6	11
2012	West Bay, NC	9	2	3	1	3	0
2012	Tampa Bay, FL	27	2	14	9*	1	0
May. 2013	Buckrow Beach,VA	1	0	0	0	0	1
May. 2013	Poquoson,VA	16	0	4	3	5	4
Aug. 2012	Sandy Hook Bay,NJ	13	0	0	0	10	3
	TOTAL	457					

* Some mothers had twins

**some samples with unknown sex

Table 2. Primer name, repeat motif, primer sequences and product size of the 95 primer pairs for amplification of microsatellite loci in the cownose ray, *Rhinoptera bonasus*.

Primer Name	Repeat Motif	Left Sequence	Right Sequence	Product Sze
Rbon_01	actc_5	TGCTTGCTCTACACCTCTCC	aAACgGCCCAGATATGATGC	119
Rbon_02	ac_29	GACCTGCTGAGTTCCTCCAG	gATCACAcagTAATTgacACAG	116
Rbon_03	aatt_5	TGCCGCTGTTGTGTACTTTC	GTCCCATGATTAGGCAAGGC	149
Rbon_04	agat_7	AATTGATCACTAAACACAACCC	GACAGACAGGCAAGCGaATG	187
Rbon_05	agat_14	AAAGCCAATTTCCCTCGGG	gGtcaAtTgcgATTtaagATAG	139
Rbon_06	acat_5	CCcTCCGATCTTACACCTACG	CCAAATGGTCCtACTCCTGC	157
Rbon_07	aaat_5	ACCAAGGCAAGATTCTTTGAC	CACAGCCCTGCATTTCCTC	104
Rbon_08	ac_28	TCATTTCCATGGATGCTGCC	gactTCaCgcCagcACAC	157
Rbon_09	ac_31	CCAAGGCCAGACTTAAGTGAC	TGAAGCAaccttgactGCTG	148
Rbon_10	ac_16	AAGTTTCATGCCTGGCCAAC	GATTGCtGAGTgGATgcTGG	177
Rbon_11	ac_16	TCCAACTTGACGCATGCATG	AGATACAGACAGTGGCCAgG	123
Rbon_12	agat_8	ACAGCCTGCCCTAGAATTCC	acgAACaAAtTTcCcatTGG	125
Rbon_13	aaat_5	TTGTCCATGAATACGACCTC	TTTGTGGCAGCAGTATAATG	88
Rbon_14	agat_5	CACGGGCTAAGAACTGTTCAG	CgaAcCCaAtTTCCcatCGG	95
Rbon_15	aagg_10	CACCGCGTTGCTCTGTAG	CAAGCAcCAGAAGCAGAGTG	160
Rbon_16	ac_27	ATCCCTTCACCCAATCTCGC	CGTCATCGTTAGGTGcgTG	110
Rbon_17	aatt_5	GTAAGAGCATAATATCGCAATG	AACTGTTCCGTGATTAGGCG	152
Rbon_18	ac_20	TGATCTGAAGCCATGGGATTTC	GCTGCCTGACCTGCTGaTG	119
Rbon_19	acat_7	AGCATTCCACATCAATTTGCAG	ACTTTGAtgctcCCagacgG	121
Rbon_20	actc_6	AGACCATTTCCTCCTCCACAC	TCTAACATcCaAGGCCTGTCC	91
Rbon_21	aggg_5	GGGAGAAGTGAGAGGTGG	ACACTCCTGATCTGCTTCCC	120
Rbon_22	ac_18	TCTGACTCTCTGTGCGGTAG	gGATAtAggaCACGaAACGCAC	186
Rbon_23	agat_5	CCTAGAGCTGTTGGTGAGGG	AACgaaccaAtTTcCCTcgG	146
Rbon_24	ac_17	TCTTTACCTGTCCATGTACAC	AAGAAGTTGCTGCAGaCgTC	103
Rbon_25	agat_5	AAACCCAATTTCCCTCGGG	AGTCCTAGCATAAATAAGGCG	103
Rbon_26	ac_13	CATAATGCAGCCACCACCAG	AcCTCACgcctATcagTCAC	189
Rbon_27	ac_21	GCTGCCTGTCAAAGTTCTACC	tcgcgtGtatgtTATCgGTG	134
Rbon_28	agat_5	ATTGGAGATCACAATGGCAC	CTGTGCACTTGTAATGCTACTG	102
Rbon_29	ac_23	TCGATGTGCTCAATGGTTGG	ctcgTAGTCTCAGCTgGGTC	163
Rbon_30	ac_18	GGGCATAAATTCAGTCTCTC	GTGAATCCTCctcTgcaAcC	143
Rbon_31	actg_5	GAAGATGGAGGTGCATAgCC	CAACATGAGATTGCTAACATTC	96
Rbon_32	agat_5	CGCTCTATTCTTGGTTGGTGC	AATGACAAAGAAGGCATGCC	175
Rbon_33	acag_5	GTCACCAAAGTCCGCATGG	AAGTCCCTTGGTCCTGATGG	130
Rbon_34	agat_7	TGGTGCGGCTGTAATGAAAC	ATGTCAGTCCATGcCTCCTC	107
Rbon_35	agcc_5	AGATGATTGTCCAACTGCTG	AGAGACTcaaccaacCCAGG	76
Rbon_36	agat_7	TGGTGCAGCTGTAACGAAAC	tCgGAGTGTGAGGTATGAtTcC	119
Rbon_37	agat_7	CGAAACCCAAATTCCCTCGG	AGGAAGCCATTAAACCCATCAG	136
Rbon_38	actc_10	TGAACGATGCAGTAGGAGGG	TGTGATGATCGAGTGTGAG	141
Rbon_39	aggc_6	CTCAACCGGCACACTCATTC	CGCtTtgcCACTCTGTCTTC	103
Rbon_40	agat_6	ACCCAATTTCACTCGGGATC	ATAAtTtcCCATTCCTGAACTC	123
Rbon_41	aagt_6	GCCTGTTCTGCGCTGTAG	gGGAGGGTGAAGAGGAAGTTC	126
Rbon_42	ac_10	AGATAACTTACTCGGAGCACAC	TCTCACTGCCTTCCCTTGTC	204
Rbon_43	aagg_18	GCACAGAAGCAGAGTGAAGC	GGGATGGAGTGCGAGGTG	203
Rbon_44	atc_9	ATGATGATGGACCCGGACAG	TGATTTCCAATTTCTACTGCGC	202

Rbon_45	ac_8	CACTCACTGAAACCCGAGTG	TGCGTGTGAAAGTGTGATGAC	202
Rbon_46	ag_8	CAGAGCTCCTAGTACCGCAG	TTCGTTTCACTTCTACCTACCC	198
Rbon_47	ag_10	GTTCCCTGAAAGAAGCCGTG	GTTCTCAACTGGCCACGTTC	196
Rbon_48	ac_29	GAACACGCCCATAATGTCCG	CCGATGCTCTCTACGGTCG	192
Rbon_49	ag_9	GGACGAGGCGGGAGTATAAC	AATATGCACTCTGTTGCCGC	187
Rbon_50	ac_13	GGTGTTGCTGAATGTACCGTAG	CTTTCACACGCTGCGACAC	186
Rbon_51	ac_29	CCATCAAACCTTGCCCGTAG	GCTCTCTGCGTCATGGTCAG	181
Rbon_52	ac_16	ACAAGGTTCAGGGTTCTTGG	TATGTGTGCCGGTGTGCC	180
Rbon_53	ac_8	TGATCTGTGGAGGGAATCGG	TCATGGTTGGTAGACACAGAC	178
Rbon_54	ag_43	GCGAGCGTTTGGGATGATTG	GTGCCTCGGTCAGTCTCG	175
Rbon_55	ag_11	ACCGTCAGTTTGCCTCTCC	TGTCTTGCTCGTCTGTTTGTC	170
Rbon_56	ac_13	AGGGAGAGACAGGTCAATGC	TCCAAGTCCAACAGAGGTCC	169
Rbon_57	ac_26	TATTGGATTCTGACGCCACG	GTCTAGCTGGCTCGGTCG	167
Rbon_58	ac_9	TGTTGGATCACAGACCTGAAAG	CGTTCTCGTCATGTCAGTTTG	167
Rbon_59	ac_15	GTGTTTCGGTGTCTGCATCC	GGTCTCAGCATACATGGACAC	166
Rbon_60	ag_8	CAGTTCCATTGTTGTGCGGG	CCTTCCCTCCACATCTCCG	165
Rbon_61	ag_25	TCCGAGAATGGGAGGAGGG	TCGGTACTTCGCTACGTCTC	164
Rbon_62	at_10	ACAGGGAGAGAGAGTAATACCC	GTTGGAACGAGTGGAGAGATG	162
Rbon_63	ac_17	TGCAGGCTGGGAGTCTAATC	ATGTAATGGATAGGCGCCCG	162
Rbon_64	ac_20	TGCCACACATTCTTCTACCC	AGGATGGTGAGGTTGAGTGG	162
Rbon_65	ag_13	TGCAGCAGAAGAGAGGGATC	GTTCAGAGGACAACAGGAAGG	162
Rbon_66	ac_8	ATAGCCCAAGTCCCTGAGTG	CATGGTGCGAGTGAGTGC	159
Rbon_67	ac_21	TGCAAGGAATTAGTCTCCTCTG	ATCGTAGCAGACCAGAACAC	159
Rbon_68	ac_11	AGATGAGATTCAGGTGCGCC	GACCGTCCTTCCAGTTTATTCC	158
Rbon_69	ac_13	AGATGAGATTCAGGTGCCCG	GACCGTCCTTCCAGTTTATTCC	158
Rbon_70	ag_21	CAACTCGCCTTGCTGTTACC	TGTGGTCAGGTCAGATGCAG	158
Rbon_71	ac_8	GTCGCACATTAGCACCTTCC	AGTCAAGTAGGAATGTGGAGTG	158
Rbon_72	ac_8	GCTGAGCTATAAACTTCAACGC	TCCACAGCCATCAAGTCCTC	157
Rbon_73	ag_8	GATGGTTTGGTTTGGAAAGGAG	TCAGTGCTGTCTCCAACTCC	154
Rbon_74	ac_8	CTCAAGGACATACACGCTGC	GCCCTAGACTAACAGCAACC	153
Rbon_75	ac_10	GAGCACATGAACACTACCACC	GTTTGTTGTTATGTGGCAGCAG	152
Rbon_76	ag_8	TCAGTTCAGTTCAGTTCGTGG	CCCTGGTTAAACGGTCACAAG	152
Rbon_77	ag_10	GCAGACGGTGGAGAAATAGTG	TCTGGAATGCTAGGGTCTCG	152
Rbon_78	agat_6	AGCATTGCCTTGGTTGGTG	ACAATTCTGGCCAGGTTAGC	152
Rbon_79	ac_9	CACATCCTAACACGACCCTTG	TTCGAGATCCCAGCGACAC	151
Rbon_80	ac_11	TGGAACAGGTAGAACAGGGC	GGGAGTGGAAAGCACAGAATC	151
Rbon_81	ac_8	GTGCGTGTGGATCCTTTGTC	CCTCAGAAGTTCACAGCACG	150
Rbon_82	ac_10	GGATTGAGCCTCTTCACCTG	TGGAGGTCAGTATTCACACCTG	150
Rbon_83	ac_32	TGTACTGGTCATAATGCATCCC	CGTCAGCTGCCTTCGTACTC	147
Rbon_84	ag_8	GGCGTTGTGATGGAATCTTAAG	CTGCGAAAGGGACAGTTACG	146
Rbon_85	ag_11	CCAATGGCGTGTAGGACAAC	GCACCTGTCCAATCATCTGC	146
Rbon_86	ac_10	TCGGGAAACAGACGGAACAC	GAATGGAGGTGGAAACGACG	146
Rbon_87	ag_26	ATGTGTTCTCCAGACCAGGC	GTCAGCGTATGGGTCAGTTG	145
Rbon_88	ag_8	GACAGTCTTAGAATCCGATGGG	AGAGTTTCAAGGGACAACAGAG	145
Rbon_89	ac_8	AGCGCCAACACTATTACAGC	TTGGACTGTGGGAGGAAACC	145
Rbon_90	ac_11	ATGGACATGTGTTAGTGCGC	CTCCAGACGTTCTCCCTCTG	145
Rbon_91	at_9	TCCAGACAGTTAACCCAGTTAC	GGAGGAATGAGACAGCTTTCG	144
Rbon_92	agat_6	TGAGTCAACCTATACCGCCAG	TCCTCCTTCCGTTTCTCCTG	144
Rbon_93	ac_8	GGTGATTTGACCGGACATGG	ACGTTGCAAATCCTCAATCCG	144

Rbon_94	agat_7	TGCCATCGAAGTTTCTGAGC	TCGCCAGATGAATGATGAATGG	137
Rbon_95	agat_6	CTATTCTTGGTTGGTGCGGC	CATCATAACAACTTTGCTGCCC	127
Rbon_96	agat_10	TGACTGGGTTCTGGGATCAC	TATTCTTTGGTTGGTGCCGC	136

Figure 1. The three mitochondrial groups (clades) present based on ND2. This represents a reduced set of the actual number of samples sequenced for visualization. Note, the samples in purple and orange are *R. bonasus*, while the samples in green correspond to the potential other *Rhinoptera* species found in the Gulf of Mexico.

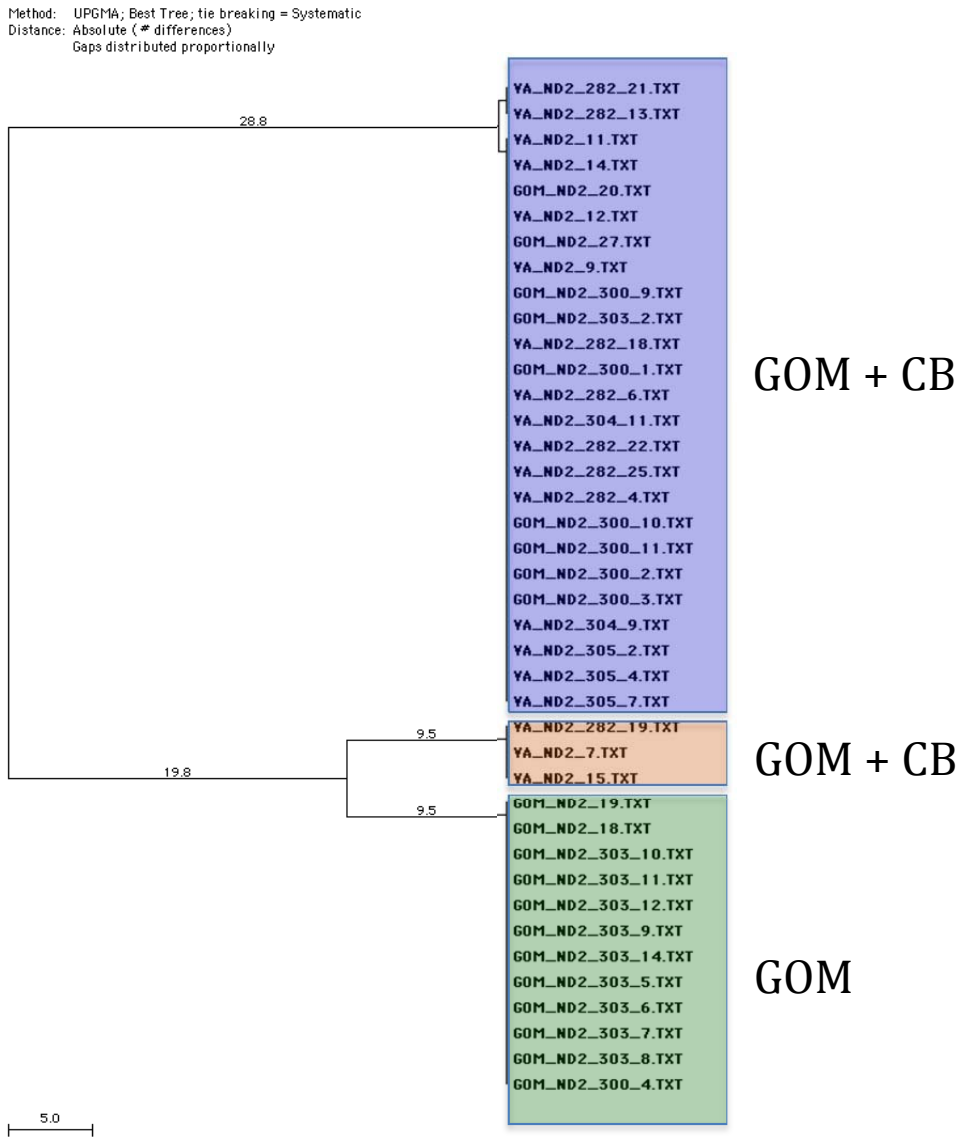

Figure 2. Comparison of phylogenetic trees of relationships among sequences resulting from mitochondrial ND2 sequences on the left and nuclear RAG1 sequences on the right. Note that the clade present only in the Gulf of Mexico remains distinct and separate from the rest of the samples when RAG1 is sequenced while the other two groups that were distinct based on ND2 (purple and orange) cannot be distinguished using RAG1 suggesting the possibility that it is not *Rhinoptera bonasus.*

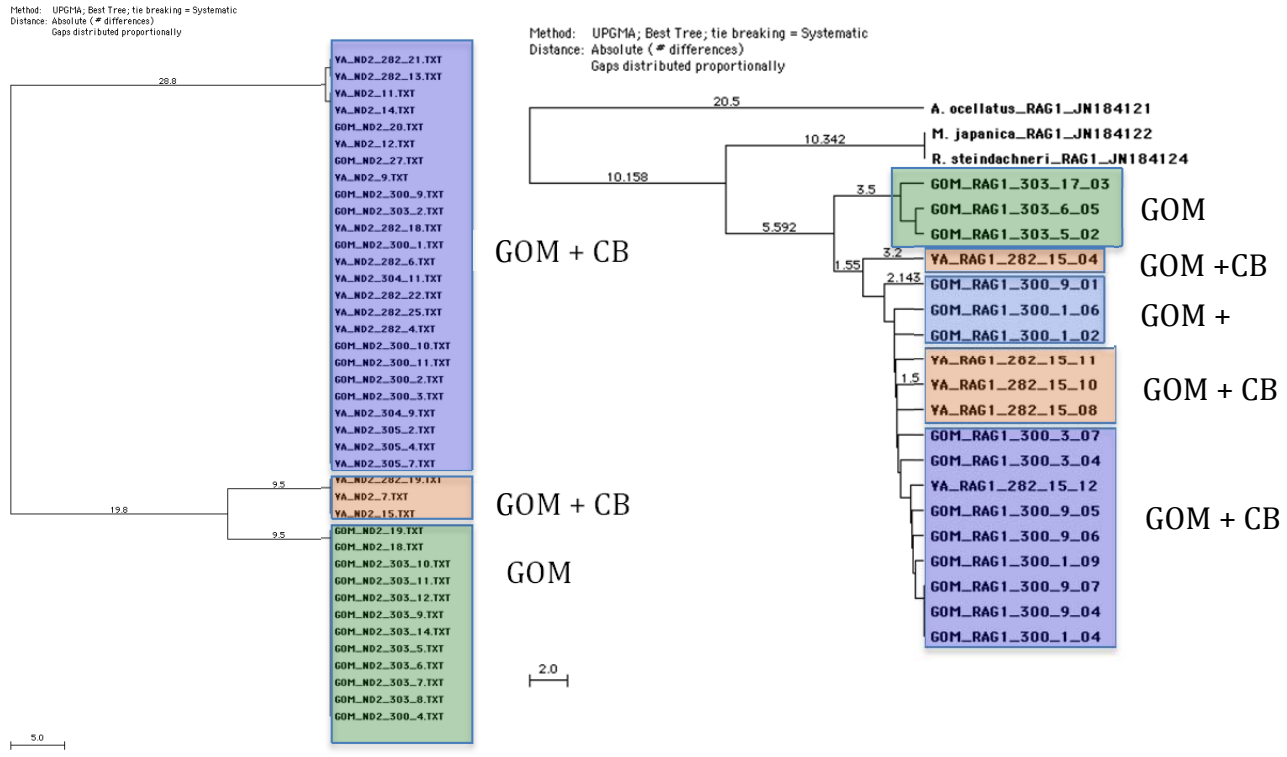

Figure 3. Phylogenetic trees of relationships among sequences when samples of *R. brasiliensis* from Brazil and samples of *R. bonasus* from Brazil and Columbia are included in the analysis of ND2 sequences.

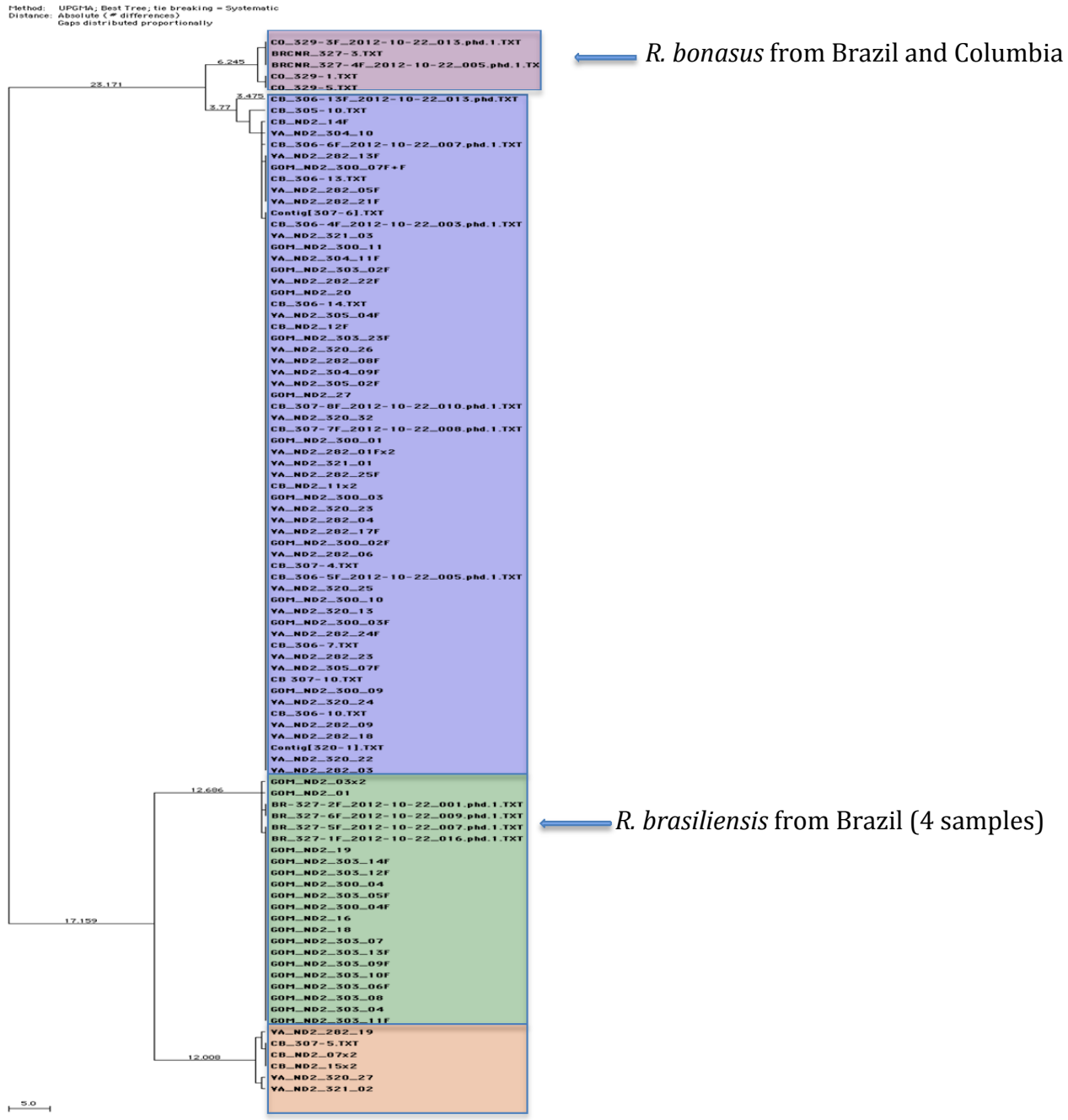

Figure 4a. Principal components analysis of samples based on microsatellite loci. Samples 8 and 5 are *R. brasiliensis* collected from Brazil and samples from the Gulf of Mexico identified as *R.* aff. *brasiliensi*s respectively. Samples 1-4 are *R. bonasus* taken from the Gulf of Mexico and U.S. east coast and samples 6 and 7 are samples of *R. bonasus* taken from Columbia and Brazil, respectively. The axes separating the samples are significant based on 10,000 permutations of the data.

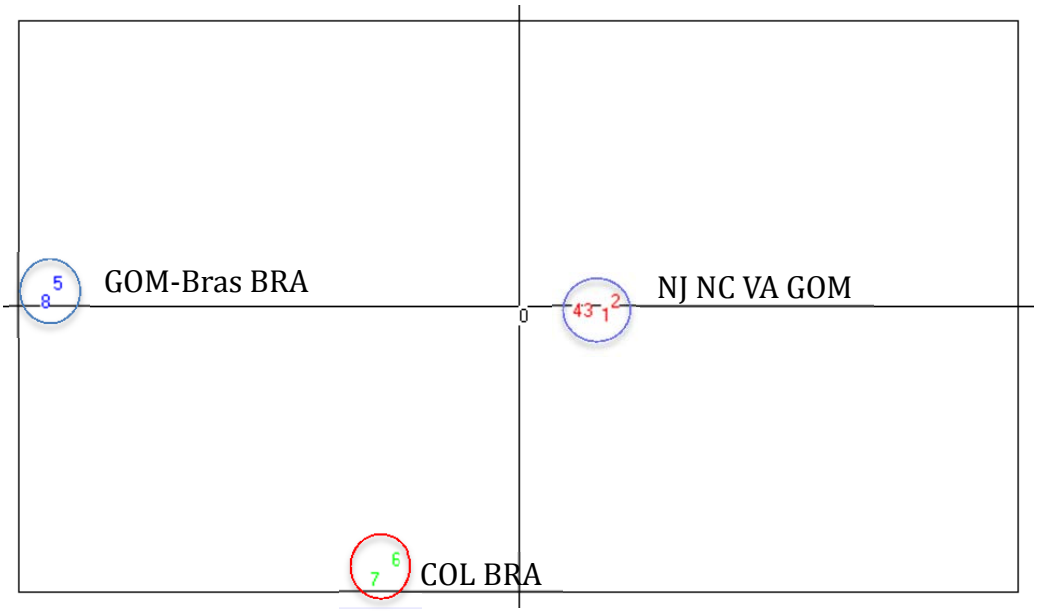
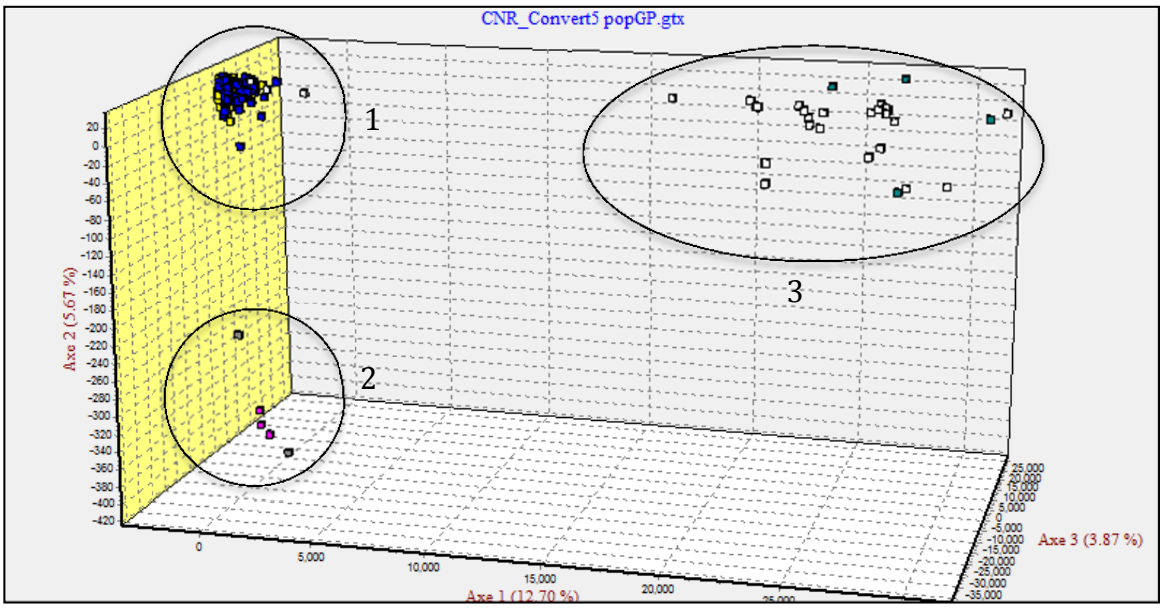
Figure 4b. Factorial correspondence analysis of samples. Group 1is comprised of *R. bonasus* taken from the Gulf of Mexico and U.S. east coast. Group 2is comprised of samples of *R. bonasus* taken from Columbia and Brazil. Group 3 is comprised of *R. brasiliensis* collected from Brazil and samples from the Gulf of Mexico identified as *R*. aff. *brasiliensis*..