

Thesis

The utility of the ITS-1 region in assessing the genetic variability
in the brine fly, *Ephydra gracilis*

Submitted by

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As a partial fulfillment of the requirements for

Thesis in Zoology

Weber State University

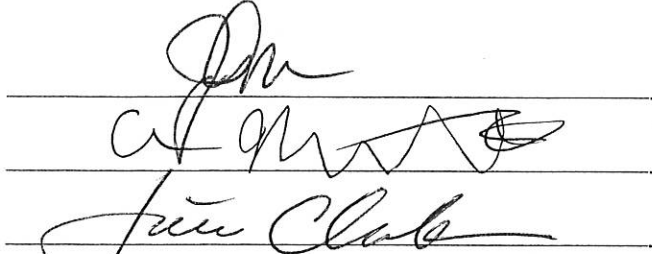
Ogden, Utah

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WEBER STATE UNIVERSITY

We hereby recommend that the thesis prepared under our supervision by Brian J. Oney entitled "The utility of the ITS-1 region in assessing the genetic variability in the brine fly, *Ephydra gracilis*" be accepted as fulfilling partial requirements for the Thesis in Zoology.

Thesis Committee



Advisor



Department Chair

Introduction

The socioeconomic value of the wetlands of the Great Salt Lake (GSL) ecosystem is priceless. The buffer zones created by the wetlands protect many areas used by humans for recreation, agriculture and housing. The wetlands are also an important nursery and staging location for many migratory bird populations, as well as a sink for pollutants. As the largest U.S. lake west of the Mississippi River, the GSL serves as one of the largest migratory bird refugia in North America.

Brine flies or shore flies (Diptera, family Ephydriidae) are located in many regions throughout the United States. Blanketing the rocks and shore in black, the flies associated with the GSL can reach estimated densities of 220 million flies per kilometer of shoreline (UDNR, 2000). Brine flies perform a critical ecological function, processing an estimated 90 million kg of organic matter annually (UDNR, 2000). As algivorous primary consumers, they are a necessary link between the primary production and upper levels of the food web of the GSL. Most notably, they are an important food source for the millions of birds that depend on the GSL.

In 1959, the railroad rock causeway was completed, and thereby separated Gunnison Bay and Gilbert Bay with the exception of two culverts for water flow, (Fig. 1). Because all the major tributaries flow into the south arm, the hydrological properties of the two arms diverged, most importantly salinity. The amount of biota is much lower in the Gunnison Bay due to the higher brine concentrations. Brine fly larvae use algal bioherms and other benthic refugia to pupate and the diet of brine flies of the GSL consists chiefly of algae, bacteria and organic detritus (UDNR, 2000), of which all are much less abundant in Gunnison Bay.

There are two principle species of brine flies associated with the GSL, *Ephydra hians* and *E. gracilis* (Fig. 2). Little is known about the precise distribution of the species or the extent to which flies of one region can interbreed with members of the same species from different areas of the GSL. *Ephydra gracilis* far outnumbers the larger *E. hians* in both arms of the GSL, however both species occur in lower numbers in the north arm, likely due to the higher physiological stresses associated with the much higher salinity (~220 ppt) of Gunnison Bay (Post, 1980). The focus of this study was an analysis of the population genetic structure of *E. gracilis*, specifically to examine whether genetic differentiation has occurred between the brine flies of the north and south arms. Of critical importance to such an investigation is the specific region of DNA to be examined. Due to the intraspecific focus, it is important to concentrate on a segment of DNA that is changing relatively rapidly over time, because the rock causeway was finished in 1959. The DNA chosen for this study is the internal transcribed spacer region known as ITS-1.

This study examined sequence variation of the ITS-1 region within two groups of *E. gracilis*, one at Rozel Point in the north arm of the lake and one from Antelope Island in the south arm. Because of the potential for interbreeding, it is expected that flies from a given group would be more genetically similar to each other than they are to flies from different groups. The objective was to determine the feasibility of using ITS-1 for studying genetic variation within and between populations of *E. gracilis*. Specific objectives include: (1) to clone and sequence the ITS-1 region from individual flies; (2) to examine the extent of variation of ITS-1 within individuals; (3) to examine the extent of intra-population ITS-1 variation; and (4) to compare intra- and inter-population genetic variation.

Materials & Methods

Fly Collection and DNA amplification

Several thousand brine flies were collected from Rozel Point and Antelope Island (Fig. 1) during the summer of 2006. A simple swoop technique with a plastic bag was used to collect flies along the shoreline of the sample sites. The flies were stored frozen. An individual fly was crushed in 50 μ l of homogenization buffer and then the mix was heated for 15-20 min at 95-100 °C in order to lyse the cells, inactivate any nucleases, and isolate DNA.

Internal Transcribed Spacer 1 (ITS-1)

ITS-1 lies between two regions that code for ribosomal RNA, the 18S and 5.8S genes (Fig. 3). The transcribed spacer region is eventually cleaved from the primary transcript and degraded. These rRNA genes themselves are highly conserved due to their importance in cellular function. However, the region between the genes, ITS-1, is noncoding and, therefore, changes relatively rapidly over time. Mutational changes include nucleotide insertions and deletions, which result in size variation of the ITS-1. For genetic studies, it is an additional, useful feature of the ITS-1 region that it is present in approximately 200 copies within the genome (Hillis and Dixon, 1998). Therefore, it is possible to assess the amount of genetic variation within an individual fly and compare it with the variation seen among different individuals. A final advantage to using this rRNA region is that it exists in nearly all eukaryotic taxa, and the ITS-1 region has been utilized in many studies of genetic variation in both vertebrates and invertebrates. Therefore, the results of this study can be compared with those of other ITS-1 studies, especially those pertaining to other insects.

Polymerase Chain Reaction

A 1 μ L portion of the DNA isolate was used as a template in the polymerase chain reaction (PCR). One of the primers (7247) used was complementary to the region at the 3' end of 18S rRNA gene and the second primer (7246) was complementary to the 5' end of the 5.8S gene (Fig. 3). The sequence of primer 7247 is 5' - CCAAACAAGGTTTCCGTAGG - 3' and of primer is 7246, CCTGCGTTCTTCATCGAC. Standard 25 μ L reactions were performed in separate reactions with DNA from each individual fly.

Reaction volumes were as follows:

15.625 μ L water
2.0 μ L dNTPs (10mM ea.)
1.25 μ L 7247 primer (5 μ M)
1.25 μ L 7246 primer (5 μ M)
2.5 μ L 10X Buffer
2.5 μ L MgCl ₂ [25 mM]
0.125 μ L <i>Taq</i> polymerase [5U/ μ L]
1 μ L DNA template

25 μ L total reaction volume

Template DNA concentrations ranged from 5 ng/ μ L to 50 ng/ μ L. One drop of mineral oil was added to each tube and the reaction was placed into a pre-heated (94°C) thermocycler. A step-cycle file with 40 cycles was used. Each cycle consisted of: one minute at 94°; one minute at 50°; one minute at 72°. Following amplification, the products were analyzed by electrophoresis through a 1% agarose gel (Fig. 5).

Vector Ligation

The PCR products were added at concentrations of 10-20 ng/ μ L to the plasmid pCR®2.1-TOPO® (Fig. 6). The reaction was incubated at room temperature for five minutes according to the TOPO® cloning reaction protocol (Invitrogen).

Bacterial Transformation and Growth

One Shot® chemically competent *E. coli* cells were inoculated with 2 μ L of TOPO® cloning reaction and were treated according to protocol from the Invitrogen TOPO® TA Cloning® instruction manual (version K2) to transform bacteria. The bacterial solution was spread onto selective growth media in amounts of 50 μ L and 150 μ L, in order to ensure properly spaced bacterial growth colonies. The growth medium was Luria-Bertani (LB) containing ampicillin in 1 μ L/1mL concentrations. X-gal was spread onto plates (40 μ L) shortly before plating the transformation reaction. X-gal is a compound that will be converted into a blue product in the presence of the plasmid alone, but remains colorless if the plasmid was successfully ligated with the insert. The blue coloring is produced by reaction of the products from the expression of the LacZ α region of pCR® 2.1-TOPO® and X-gal. The ligation of the PCR product into the vector disrupts the expression of this gene rendering the colony white. False positives occur due to the loss or removal of the 3' deoxythymidine, also disrupting LacZ α 's expression. The agar plates of spread bacterial solution were grown 10-14 hours in an incubator at 37° C. A single colony stemming from a single transformed bacterium was inoculated into 2 mL of selective (ampicillin) liquid growth medium and cultured 16-20 hours in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co., Inc) at 37°C.

Plasmid Isolation

Plasmids were isolated from the bacterial culture using a standard protocol and screened to see if the plasmid had indeed joined with the PCR product to form a recombinant plasmid. As an inexpensive screening for presence of the insert, plasmids were isolated and endonucleased with *EcoR* I as per protocol (Table 1).

After screening, a Wizard mini-prep kit (Promega Corp.) was used to isolate plasmids according to the manufacturer's protocol. The plasmids were purified, screened for the presence of adequate concentrations (Fig. 8) and subsequently sequenced with an ABI Prism automated system.

Sequence Analysis

Sequence analyses and manipulations were performed with the computer programs MacVector and PAUP 4.0 (Phylogenetic Analysis Using Parsimony). PCR®2.1-TOPO® has two promoter regions (T7 and SP6, Fig. 6) that allow the inserted DNA to be sequenced from either direction. Thus, a sequence obtained must be examined to determine its orientation. As sequences were received they were examined to determine which of the primers and accompanying DNA strand had been sequenced. If the sequenced DNA began with the 7247 primer, it and upstream DNA were deleted from the sequence. If the sequenced DNA began with the 7246, the reverse and complement of the sequence were acquired using the MacVector program. Sequence alignments were done using the online program, ClustalW. The resulting alignments were used to perform a quantitative sequence-difference analysis. This difference consisted collectively of insertions, deletions, substitutions, and haplotypic positions.

Haplotype Analysis

Through sequence analysis it is possible to identify different haplotypes. Comparing only nucleotides at haplotypic positions, sequence differences were tabulated (Table 2). Entire sequences were compared to each other using the PAUP 4.0 program. Gross sequence differences were changed to percent differences, averaged for each case comparison and plotted (Fig. 9).

Results

The ITS-1 region that was amplified and sequenced comprised an average of 666 nucleotide base pairs (bp) in all flies (Fig. 5). A total of 53 sequences were obtained, 31 from the Antelope Island population, and 22 from Rozel Point. When the sequences were aligned, occasional insertions and deletions were seen (see Appendix). These corresponded to mutational events that occurred in various copies of the ITS-1 region. Cumulatively among the ITS-1 sequences, there were six single nucleotide polymorphisms (SNPs), a double nucleotide polymorphism, and an adenosine microsatellite locus. There were numerous instances in the analyses, where two sequences were 0% different, that is 100% identical.

Through sequence analysis it is possible to identify different haplotypes. Each haplotype represents a unique pattern of nucleotides that are shared by individuals as a result of inheritance from a common ancestor. Comparing only nucleotides at haplotypic positions, sequence differences were tabulated (Table 2). These differences are not stochastic, but rather they represent a different haplotype. Occuring 32 times in the *E. gracilis* genome, the summarized haplotype sequence X was shared by 12 individuals

from Rozel Point and 20 individuals from Antelope Island (Table 2). As summarized in Fig. 9, there is very little discrimination within or among individuals from the two populations of *E. gracilis* sampled and the overall percentage of shared sequences is very high (77%) (Fig.10).

Discussion

This study reveals very little discrimination within or among individuals from the two populations of *E. gracilis* sampled. This lack of discrimination can be explained in three ways. (1) Along the eastern and northern shore of the Great Salt Lake, *E. gracilis* represents a single, continuous population that exhibits little genetic differentiation. Since there are few published data on brine fly biology, it remains to be determined if this hypothesis is consistent with brine fly distribution. (2) Due to a relatively small sample size, these preliminary results may not reflect the overall pattern of variation that exists in this population, which is quite large, consisting of billions of individuals. (3) The ITS-1 region is an inappropriate DNA sequence for trying to answer the questions that were the subject of this study, because it is too variable. Even though ITS-1 has proven useful in a number of other similar studies, in a variety of organisms, no DNA region is appropriate to assess genetic variation in all taxa.

Based on a number of previous studies, the ITS-1 region is useful when examining intraspecific genetic differentiation. Vogler and DeSalle (1994) also found a high degree of sequence variation within individuals, but using a similar sequence analysis program they were able to deduce phylogenetic relationships among populations of the tiger beetle, *Cicindela dorsalis*. Presa et al. (2002) examined the utility of the ITS-1 region at a very large geographic scale and found it to also be useful in describing

relationships between populations of the brown trout, *Salmo trutta*. Performing similar analyses on the ITS-1 region of grasshopper, *Chorthippus parallelus*, Parkin and Butlin (2004) suggested that the presence of variation of the ITS-1 region within individuals was mostly due to intrachromosomal gene conversion and they were able to postulate when two geographically distinct populations diverged. Parkin and Butlin (2004) sequenced a total of 30 ITS-1 clones from 4 populations of *Chorthippus parallelus* scattered throughout the Iberian Peninsula. Presa et al. (2002) sequenced 103 ITS-1 clones and found 37 haplotypes that is, an original sequence differing significantly not as a result of mutational changes but inherited differences in sequence composition. Each of these studies observed a relatively large amount of ITS-1 sequence variation.

All of the ITS-1 studies cited above sampled sites far removed from each other (>1000 km). In this large distance there were many formidable, insuperable barriers to movement of the study species. In contrast, the two populations of brine flies sampled for this study are approximately 80 km apart with no comparable barriers separating them. Based on what is known about their ecology and with an adult lifespan of 3-5 days, brine flies mate shortly after emerging from the puparium and it is likely that they would seek the nearest mate. It is highly unlikely that a fly would traverse the entire distance of the lake. However, it is possible that wind and water currents transport adults and larvae throughout the lake. The water flow between the two arms is relatively minimal (Gwynn, 2007) but could be sufficient to transport larvae to the Rozel Point site, although it is questionable whether the larvae would tolerate such a large salinity change. The most likely explanation for movement of individuals is wind currents transporting adult flies around the lake. Due to the density of the *Ephedra* along the shoreline, the flies most

likely constitute a continuous single population surrounding the GSL supporting hypothesis (1). Thus, any mutations or variation within the genome of one fly could be propagated throughout the entire population, thereby homogenizing DNA sequences over time.

When unique mutations were removed from the analysis, phylogenetically informative nucleotide positions are left, that is, positions where two or more flies share the same nucleotide, which is different from other sequences. Parkin and Butlin (2004) used the information given from these SNPs, calling them within individual polymorphisms (WIPs), to draw their conclusions on population structure. There were 6 of these positions observed in the ITS-1 region (Table 2 & Appendix). The sequence of these positions represent a haplotype, a strictly inherited sequence that excludes mutations. The large percentage of shared haplotypes (Fig.10) strongly suggests that the two sample groups have a common ancestor, again supporting hypothesis (1).

When compared to other studies, it can be said that the total number of ITS-1 regions sequenced in this study (53) should be sufficient to reveal any genetic differentiation between the two sample groups if indeed they are different populations. In contrast to this study other studies, which also found a high degree of intra-individual ITS-1 sequence variation, were able to uncover population structure. This is likely due to the extended geographic range over which samples were collected. Thus, hypothesis (2) above seems unlikely, although more extensive sampling encompassing other regions of the lake inhabited by *E. gracilis* may provide additional evidence for or against this idea.

Harris and Crandall (2000) found ITS-1 to be phylogenetically uninformative when compared previous data from mtDNA in different populations of the crayfishes

Orconectes spp. However, Presa et al. (2002) found ITS-1 phylogenies to be congruent with those constructed from mtDNA. To further examine hypothesis (3), the results of sequence comparisons and haplotype distribution from another gene could be compared to those obtained here with ITS-1. If both genes show the same pattern, i.e., no differentiation among groups, this would argue for hypothesis (1) and against hypothesis (3). Genes that could be used include mitochondrial genes, such as *cytochrome b* or *cytochrome oxidase* subunit I, which have been useful for examining within-species variation (Cognato and Sperling, 2000; Castresana, 2001).

When the overall results of this study are compared to those described above, the results are quite similar. There is considerable sequence variation among the different copies of the ITS-1 regions within the same individual. When these sequences are compared to those from other individuals, the extent of variation is of a similar magnitude. The large majority of shared haplotypes (77%) of the flies from both groups suggests that genetic exchange is taking place between the sample groups. Therefore, hypothesis (1) provides the most probable explanation of the data. If this is the case, the ITS-1 region appears to be a suitable DNA sequence for revealing genetic variation and any associated biological changes of the study organism. The two groups of brine flies that were sampled belong to the same population, which seems to be relatively unaffected by the human-induced changes to the GSL ecosystem. Obtaining sequences of the mtDNA genes *cytochrome b* or *cytochrome oxidase* subunit I would contribute more information about the extent of genetic differentiation among populations of *E. gracilis*.

Acknowledgments

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Table 1

This table highlights the protocol used as an inexpensive plasmid DNA isolation.

1. Transfer 1.5 ml of the overnight culture to a microcentrifuge tube and pellet cells at 10,000 RPM, 2 min.
2. Remove the supernatant completely and resuspend the cells in 300 μ L STET buffer. Drag the tube across a rack approximately ten times. It is important that the cells are completely resuspended.
3. Add 25 μ L lysozyme (10 mg/ml) and mix by inversion.
4. Immediately place tube in a boiling water bath and incubate 45 sec.
5. Spin 10,000 RPM, 5 min. The pellet contains cellular debris and chromosomal DNA; the supernatant contains plasmid DNA and RNA. Remove debris with a toothpick.
6. Add 350 μ L isopropanol to the supernatant, mix well, and spin 10,000 rpm for 5 min.
7. Wash pellet once with 70% ethanol. Pellet DNA again.
8. Remove ethanol completely and let pellet dry for 10 min.
9. Resuspend DNA in 20 μ L TE buffer; store at 4°C (short term) or -20°C (long term).
To check for the presence of an insert in the plasmid, perform a restriction enzyme digest. Be sure to include a control plasmid with no insert for comparison.
10. Remove 2 μ L of the each plasmid DNA and combine with 8 μ L restriction mix (0.2 μ L RE, 1 μ L 10x buffer, 6.8 μ L H ₂ O). Mix well and incubate 37°C for 1 hour.
11. Analyze the digested DNA on a 1% agarose mini-gel (Figure 7).

Table 2

The summarized nucleotides and their respective positions in the ITS-1 region represent different haplotypes. All nucleotide positions mark nucleotides that were inherited. Sequences sharing a letter to the right are identical.

Position in ITS1	78	102	121	122	184	259	428	Haplotype
AI1A	C	A	C	T	A	A	G	Y
AI1B	C	A	C	T	A	A	G	Y
AI1C	T	A		T	A	A	G	
AI2A	T	A		T	C	G	A	X
AI2B	T	A		T	C	G	A	X
AI3A	T	A		T	C	G	A	X
AI3B	C	A	C	T	C	G	A	
AI3C	T	A		T	C	G	G	Q
AI4A	T	A		T	C	G	A	X
AI4B	T	A		A	C	G	A	W
AI4C	T	A		T	C	G	A	X
AI5A	T	A		T	C	G	A	X
AI5B	T	A		T	C	G	A	X
AI5C	T	A		T	C	G	A	X
AI6A	T	A		T	C	G	G	Q
AI6B	T	A		T	C	G	A	X
AI7A	T	A		T	C	G	A	X
AI7B	T	A		T	C	G	G	Q
AI7C	T	A		T	C	G	A	X
AI8A	T	A		T	C	G	G	Q
AI8B	T	A		T	C	G	A	X
AI9A	T	A		T	C	G	A	X
AI10A	T	A		T	C	G	A	X
AI10B	T	A		A	C	G	A	W
AI10C	T	A		T	C	G	A	X
AI10D	T	A		T	C	G	A	X
AI11A	T	A		T	C	G	A	X
AI11B	T	A		T	C	G	A	X
AI11C	T	A		A	C	G	A	W
AI11D	T	A		T	C	G	A	X
AI11E	T	A		T	C	G	A	X
RP1A	T	A		T	C	G	G	Q
RP1B	T	A		T	C	G	A	X
RP2A	T	A		T	C	G	A	X
RP3A	T	A		T	C	G	A	X
RP3B	T	A		T	C	G	A	X
RP3C	C	T	C	T	A	A	G	
RP4A	T	A		T	C	G	A	X
RP4B	T	A		T	C	G	A	X
RP4C	T	A		T	C	G	A	X
RP5A	T	A		T	C	G	A	Z
RP5B	C	A	C	T	A	A	G	Y
RP5C	C	A	C	T	A	A	G	Y
RP5D	C	A	C	T	C	G	A	
RP6A	T	A		T	C	G	A	X
RP6B	T	A		T	C	G	A	Z
RP6C	T	A		T	C	G	A	X
RP7A	T	A		T	C	G	A	X
RP7B	C	T	C	T	A	A	A	U
RP7C	T	A		T	C	G	A	X
RP8A	C	T	C	T	A	A	A	U
RP8B	T	A		T	C	G	A	X
RP9A	T	A		T	C	A	A	

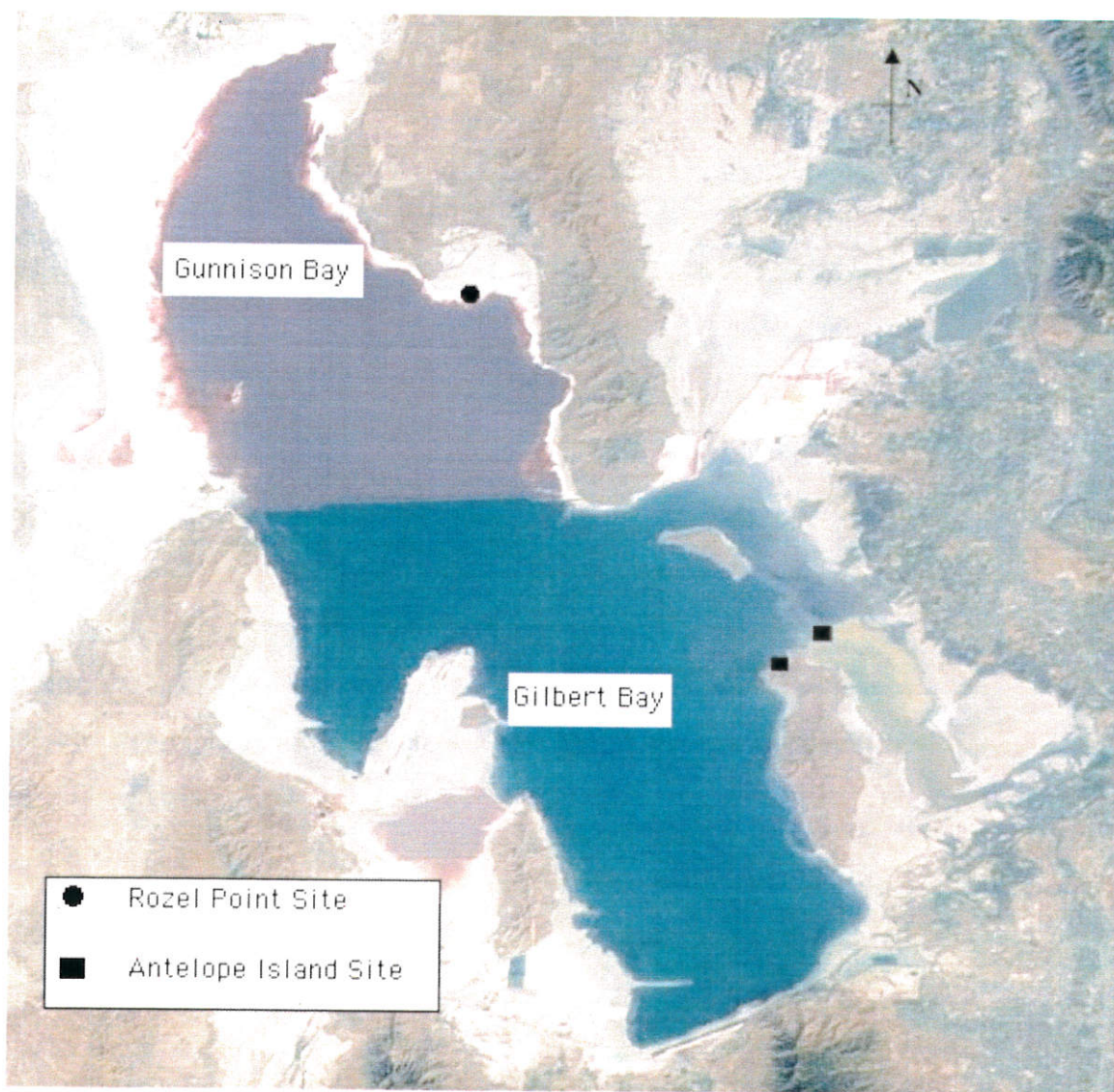


Fig. 1.—The sampling sites used in this study. Satellite picture taken from www.geology.utah.gov



Fig. 2.—The subject of this study is the gray brine fly, *Ephydra gracilis*.

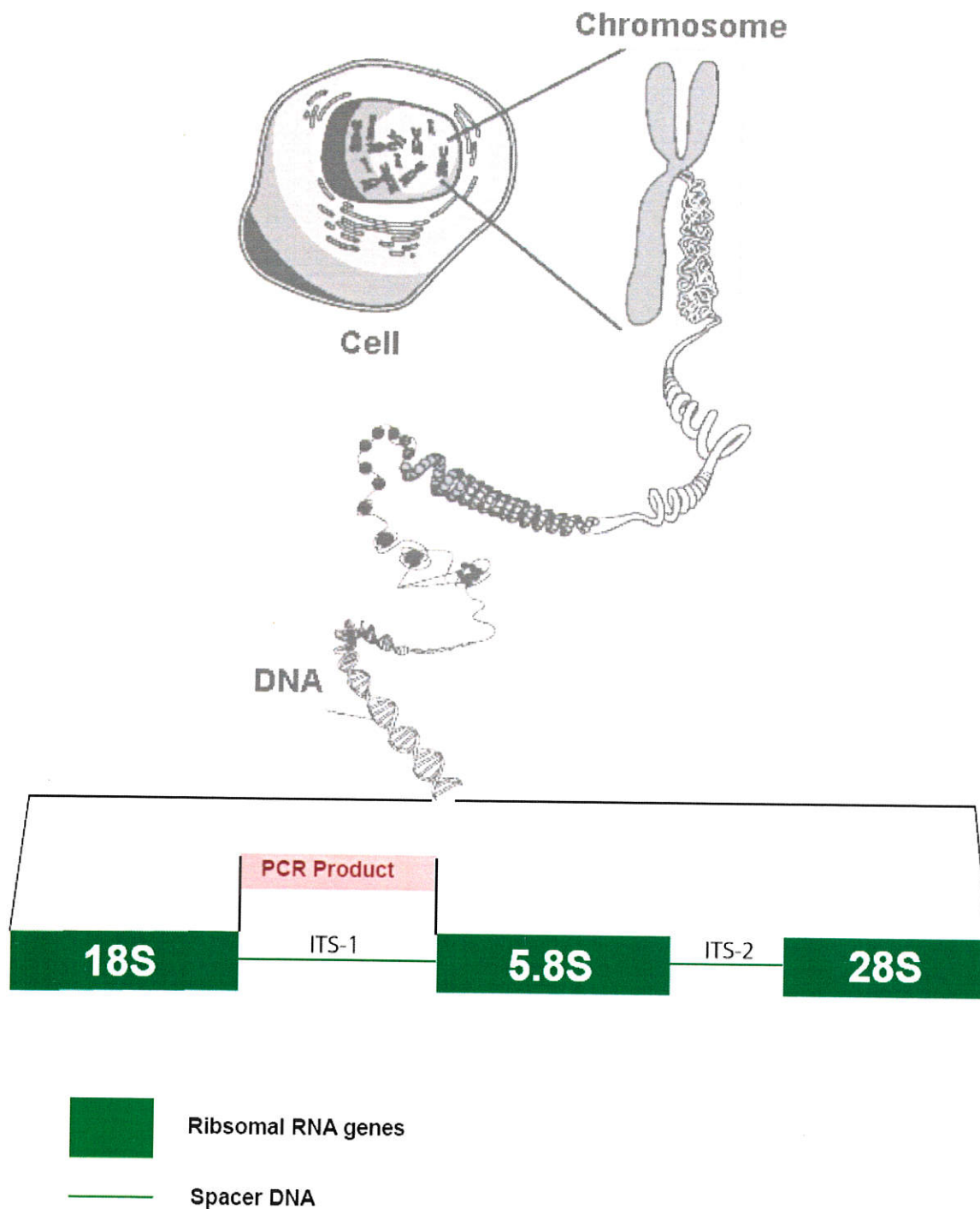


Fig. 3.—Schematic representation of the ITS-1 region.

The ITS-1 region lies between two genes that code for rRNAs. The ITS-1 region of the brine fly *E. cinerea* comprises, on average, 666 nucleotide base pairs, but varies greatly between species. The relative location of the PCR product is show in pink.

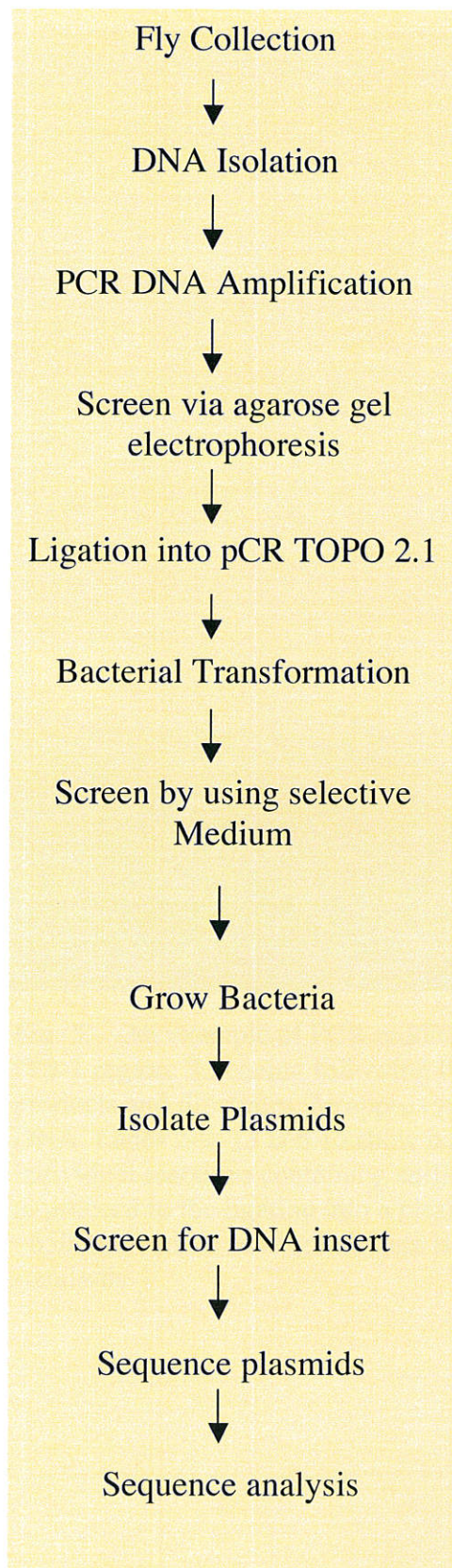


Fig. 4.—Schematic representation of the process undertaken to obtain the data in this study.

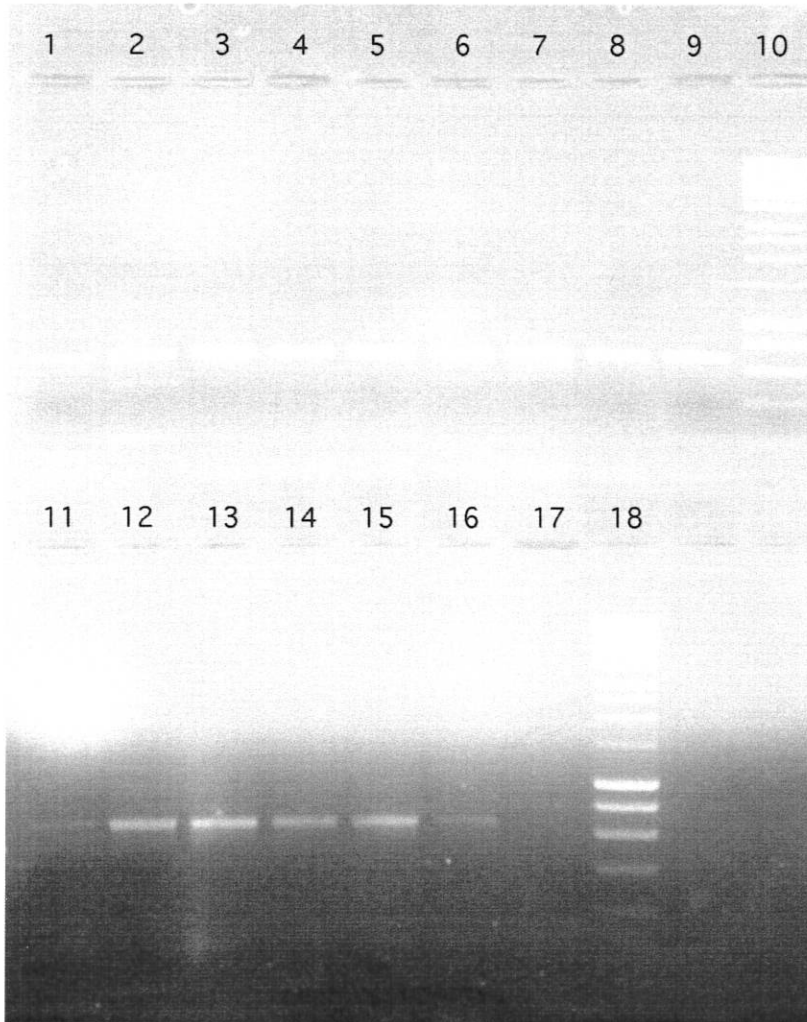
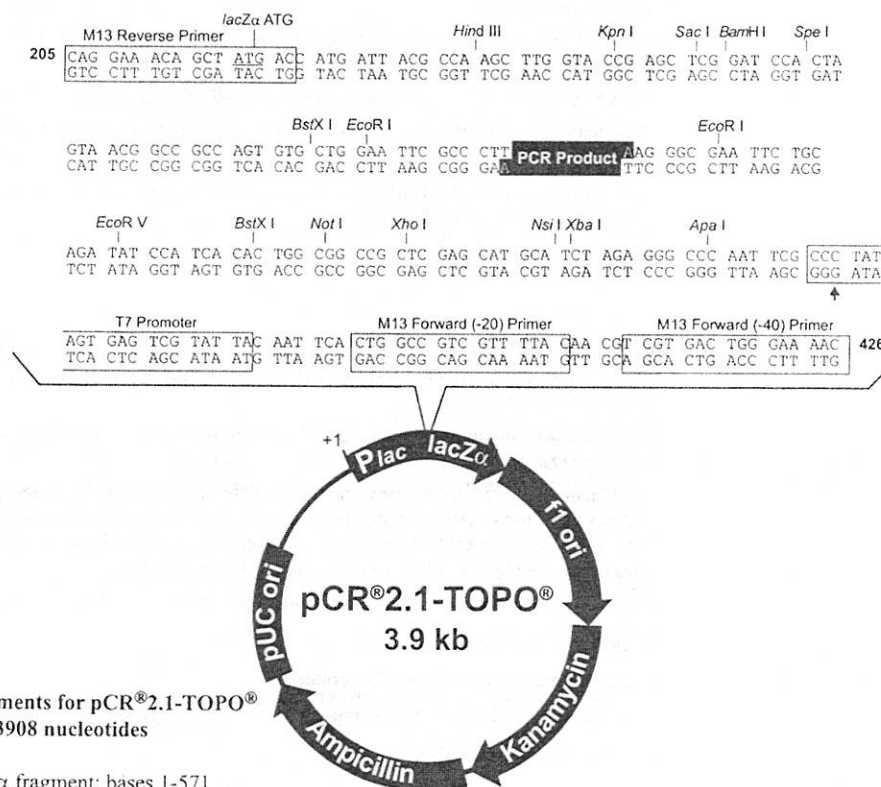


Fig. 5.—An example of PCR products associated with the ITS-1 region. Screening following PCR visualized the products and illustrates the approximate concentrations of DNA. Lanes 2-9, 13 and 15 show bands that came from individual reactions containing sufficient concentrations DNA to proceed to the ligation into a plasmid vector. The bands are ~650 nucleotides in length. Lanes 10 & 18 contain size standards.

Map of pCR®2.1-TOPO®

pCR®2.1-TOPO® Map

The map below shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase. For the full sequence of the vector, you may download it from our web site or call Technical Service (page 19).



Comments for pCR®2.1-TOPO® 3908 nucleotides

LacZα fragment: bases 1-571
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
M13 Forward (-40) priming site: bases 411-426
f1 origin: bases 548-962
Kanamycin resistance ORF: bases 1296-2090
Ampicillin resistance ORF: bases 2108-2968
pUC origin: bases 3113-3786

Fig. 6.—Map of the cloning vector, pCR®2.1-TOPO® vector plasmid (image taken from the Invitrogen K2 protocol) used for this study. The plasmids possesses an origin of replication, an ampicillin resistance region, an *EcoR* I endonuclease cleavage site, the LacZα operon, and the SP6 and T7 promoter regions.

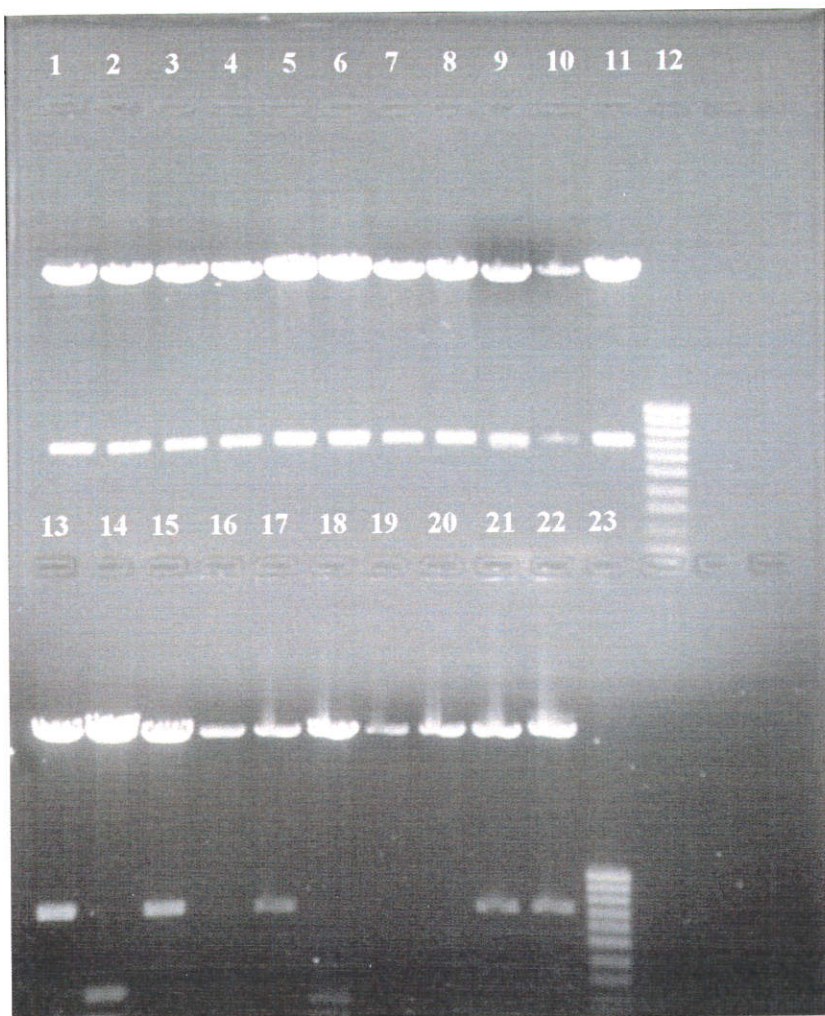


Fig. 7.—Example of recombinant plasmids containing ITS-1 inserts. Screening the plasmids (~3908 bp) for the inserted DNA (~650) revealed its presence (lanes 1-11, 13, 15, 17, 21, and 22) and its absence (lanes 16, 18, 19, and 20). The bands at ~200 bp in lanes 14 and 18 are artifacts. Lanes 12 and 23 contain size standards.

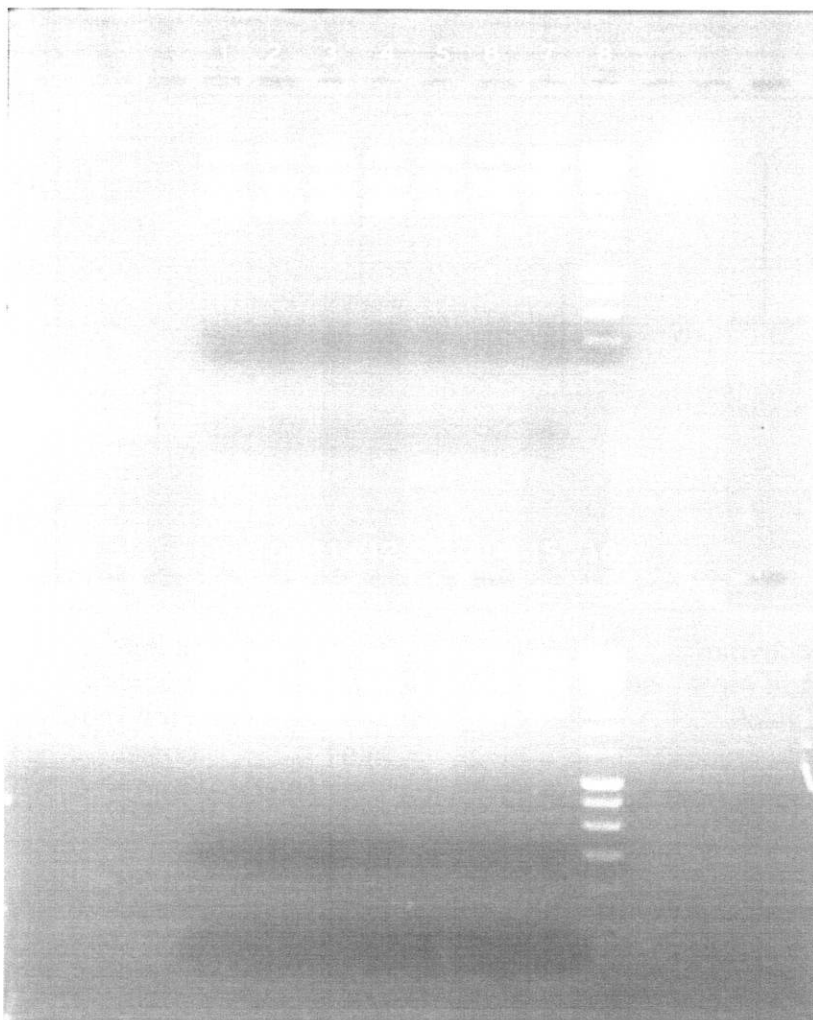


Fig. 8.—Determining the concentration of plasmids prior to sequencing. Lanes 1-7 and 9-15 show recombinant plasmids in concentrations adequate for sequencing. Lanes 8 and 16 contain a DNA size standard.

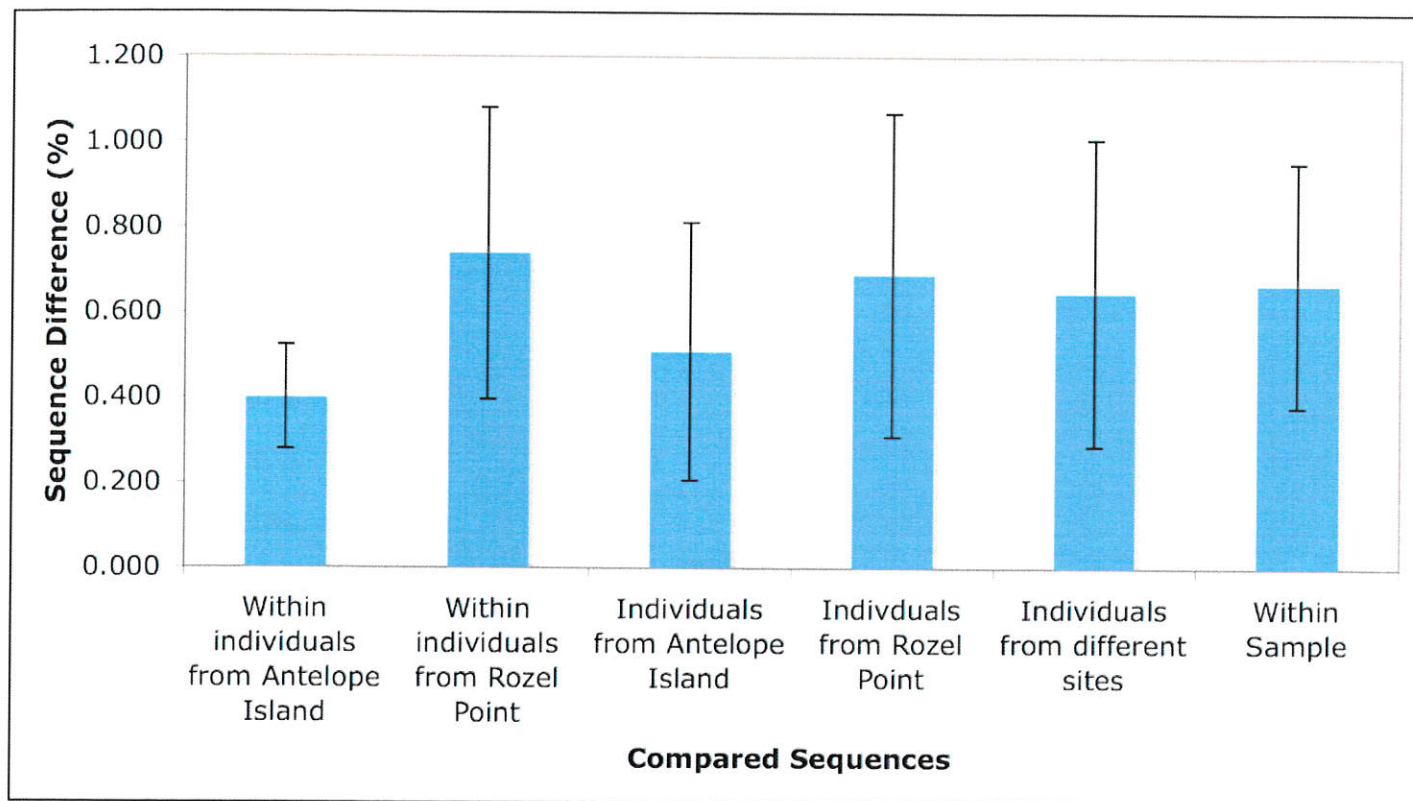


Fig. 9.—A summary of ITS-1 sequence differentiation with standard deviation bars. Complete sequences were included in each of the respective comparisons.

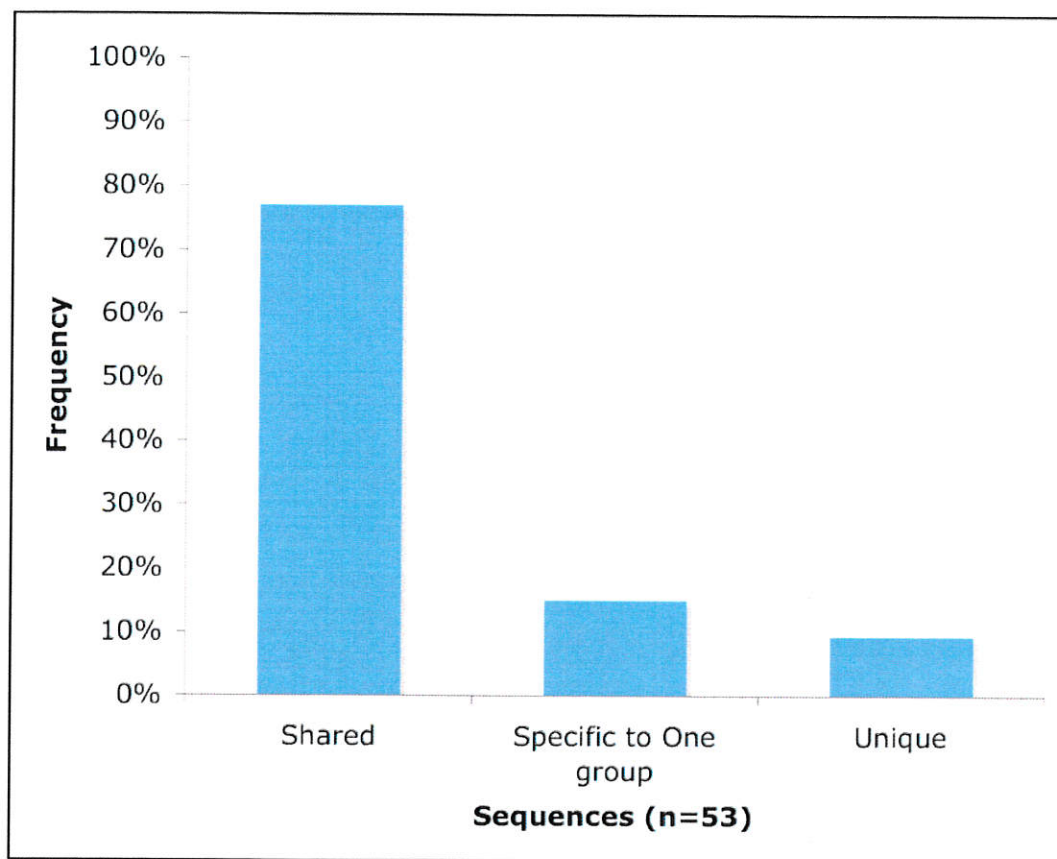


Fig. 10.— Distribution of ITS-1 haplotypes. The frequency of haplotypes common to both sampled groups is high (40 shared sequences). Some haplotypes were unique to a group (8 sequences total). Some haplotypes were unique only to an individual (5 sequences)

Appendix Alignment of ITS-1 sequences from *E. gracilis*. Sequence identities are marked by a
*. Nucleotide positions that represent a different haplotype are marked by a #, whereas
positions that are different and represent unique mutations lack a marking.

[illegible]

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[illegible]

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AI1A	ATATTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI1B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI1C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI2A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI2B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI3A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI3B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI3C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI4A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI4B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI4C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI5A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI5B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI5C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI6A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI6B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI7A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI7B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI7C	ATATTTGTGGAGCGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI8A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI8B	ATATTTGTGGAGTGNATTcantgaanaatctnttgagcttgtaaacaattttcgttgcac
AI9A	ATATTTGTGGAGTGTATTCAATGAANAATCTATTGAGCTTGNTAAACATTTTCGTTGCNC
AI10A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI10B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI10C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI10D	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI11A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI11B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTCAAACATTTTCGTTGCAC
AI11C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI11D	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
AI11E	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP1A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP1B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP2A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP3A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP3B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP3C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP4A	ATATTTGTGGAGTGTATGCAATGAAGAATCTATTGGGCTTGTTGAACATTTTCGTTGCAC
RP4B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP4C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP5A	ATATTTGTGGAGTGTATTCAATGGAGAATCTATTGAGCTTGTTAAACATTTTCGCTGCAC
RP5B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP5C	ATATTTGTGGAGTGTATTCAATGAANAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP5D	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP6A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP6B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP6C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP7A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP7B	ATATTTGTGGAGTGTATTcgaatgaagaatctattgagcttgtaaacaattttcgttgcac
RP7C	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP8A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP8B	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
RP9A	ATATTTGTGGAGTGTATTCAATGAAGAATCTATTGAGCTTGTTAAACATTTTCGTTGCAC
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[illegible]

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AI1A AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI1B AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI1C AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI2A AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI2B AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTMTAAGCGGTGGATCACTTGGC
AI3A AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI3B AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI3C AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
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AI4C AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI5A AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI5B AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI5C AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI6A AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTWTAAGCGGTGGATCACTTGGC
AI6B AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
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AI7B AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI7C AATTGACATTTATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI8A AATTGACATTAACATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI8B AATTGACATTAATTCAGANCAAAAANCAAAAATATCNCNCTNANCGNGGATCNCCTTGNC
AI9A AATTGACATTAATTACAN-CACAAAACANANATATCACTCNAAGCGGTGGATCACTTGGC
AI10A AATTGACATAAATTAATAAATAAAAAACAAAAATATCACTCTAANCGNGGATCACTTGGC
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AI10D AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
AI11A AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
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RP1A AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
RP1B AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
RP2A AATCGACATTAATTAATAAATAAAAAACAAAAATATCACTYTAAGCGGTGGATCACTTGGC
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RP3B AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
RP3C AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
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RP8B AATTGACATAAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGGGATCACTTGGC
RP9A AATTGACATTAATTAATAAATAAAAAACAAAAATATCACTCTAAGCGGTGGATCACTTGGC
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AI1A	TCATGG-----
AI1B	TCATGG-----
AI1C	TCATGG-----
AI2A	TCATGG-----
AI2B	TCATGG-----
AI3A	TCATGG-----
AI3B	TCATGGGTC----
AI3C	TCATGG-----
AI4A	TCATGG-----
AI4B	TCATGG-----
AI4C	TCATGG-----
AI5A	TCATGGGTCGAT-
AI5B	TCATGGGTCGATG
AI5C	TCATGG-----
AI6A	TCATGG-----
AI6B	TCATGG-----
AI7A	TCATGG-----
AI7B	TCATGG-----
AI7C	TCATGG-----
AI8A	TCATGG-----
AI8B	TCATGG-----
AI9A	TCATGG-----
AI10A	TCATGG-----
AI10B	TCATGGGTCGATG
AI10C	TCATGG-----
AI10D	TCATGG-----
AI11A	TCATGGGTCGATG
AI11B	TCATGG-----
AI11C	TCATGG-----
AI11D	TCATGGGTCGATG
AI11E	TCATGGGTCGATG
RP1A	TCATGG-----
RP1B	TCATGG-----
RP2A	TCATGG-----
RP3A	TCATGG-----
RP3B	TCATGG-----
RP3C	TCATGG-----
RP4A	TCATGG-----
RP4B	TCATGG-----
RP4C	TCATGG-----
RP5A	TCATGG-----
RP5B	TCATGG-----
RP5C	TCATGG-----
RP5D	TCATGG-----
RP6A	TCATGG-----
RP6B	TCATGG-----
RP6C	TCATGG-----
RP7A	TCATGG-----
RP7B	-----
RP7C	TCATGG-----
RP8A	TCATGG-----
RP8B	TCATGG-----
RP9A	TCATGGGTCGATG