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Hands-on Molecular Biology Research in the Undergraduate Curriculum

Immo A. Hansen[1,2,3], Angus L. Dawe[1,2], and C. Donovan Bailey[1]

New Mexico State University, [1]Department of Biology, [2]Molecular Biology Program, [3]Institute of Applied Biosciences, MSC3 AF, Las Cruces NM 88003 USA
(immoh@nmsu.edu; dawe@nmsu.edu;dbailey@nmsu.edu)

In conjunction with an HHMI funded *Undergraduate Research Scholars Program*, we developed an intensive molecular biology research lab designed to introduce students to lab standards, widely used molecular biology techniques, and bioinformatics analyses within the context of two seven week experiments. The biodiversity component of the course involved the design of 18S rDNA specific PCR primers and the extraction of microbial DNA from environmental samples (e.g., river sludge, soil samples, and animal stool samples). Students then used PCR to amplify the diversity of 18S sequences present in their environmental extract and applied ligation, bacterial transformation, DNA sequencing, and phylogenetics to place sequences recovered from the experiment in the context of the tree-of-life. The second half of the course involved a reverse genetics experiment where students identified genes of interest in the published genome sequence of the Northern House Mosquito and subsequently cloned partial cDNAs of these genes. The students then used RNAi-mediated gene silencing to study the function of these proteins in a mosquito bioassay. Student activities also involved developing and presenting short lectures on various molecular biology techniques. These experiences provide undergraduate scholars with hands-on knowledge in experimental design, data gathering and documentation, and data analysis.

Keywords: Molecular Biology, biodiversity, PCR, phylogenetics, reverse genetics, RNA interference

Introduction

Here we present the details and objectives behind a "Molecular Research Methods" undergraduate laboratory course carried in the Department of Biology at NMSU. The curriculum focuses on the training of undergraduate student cohorts in molecular laboratory practices, including basic lab chemistry, standard lab techniques, and documentation. Through active involvement in experimental planning and each component of two seven week long experiments, we immerse students in the scientific method and the practice of molecular biology. The majority of students involved in the course are also in NMSU's HHMI *Undergraduate Research Scholars Program*, which guides students into research laboratories and allows them to apply their newfound knowledge and skills to independent research projects that culminate in an honors thesis experience.

This course takes advantage of the expertise of multiple faculty. As a result, we employ two sequential seven week research projects that overlap in aspects of the molecular biology involved, offering significant reinforcement and practice of each technique while progressing toward successively more complex techniques in molecular biology. The version of the course presented here includes a molecular-based assessment of microbial biodiversity (*Part I*) along with a reverse genetics experiment characterizing the function of aquaporin water transporters in the southern house mosquito (*Part II*). The course format involves two three hour lab sections per week with lecture and extensive discussions interspersed during breaks between experimental steps.

Student Outline

Part 1: Discovering and Characterizing Microbial Biodiversity in Environmental Samples

Introduction

With at least 1.2 million species currently described on the planet and estimates ranging to more 10 million species awaiting discovery, surveys of microbial biodiversity have come the forefront of studies focused on species discovery and biodiversity (e.g., Cheung *et al*., 2010; Floyd *et al*., 2005; Mora *et al*., 2011). These experiments typically use DNA extracted from environmental samples (water, soil, air, etc.) for amplification of conserved sequences that are compared and used to estimate the number of species and to characterize the diversity of microscopic organisms represented within the sample. Nuclear ribosomal genes are among the few DNA sequences for which conserved primer sites can be identified that amplify sequences from all living organisms. These permit biodiversity estimates on a scale not possible prior to the advent of polymerase chain reaction (PCR), cloning, DNA sequence analysis, and the availability of reference material in well sampled online databases (e.g., GenBank).

The first seven weeks of the course incorporates a DNA sequence-based survey of microbial biodiversity, with emphasis on eukaryotic organisms. These surveys have involved the use of DNA sequences from the 18S subunit of the ribosomal repeat, which have been widely applied to survey cryptic microbial biodiversity in a wide range of environments (e.g., Cheung *et al*., 2010; Floyd *et al*., 2005). Below we outline the primary topics covered along with the key concepts introduced and/or reinforced through each step of the experiment. This segment involves DNA extractions from environmental samples, gel electrophoresis, PCR and primer design, the cloning of PCR products, DNA sequencing, and the bioinformatics associated BLAST searches along with stand-alone phylogenetic analyses used to characterize species diversity recovered from student experiments (Fig. 1 and Table 1).

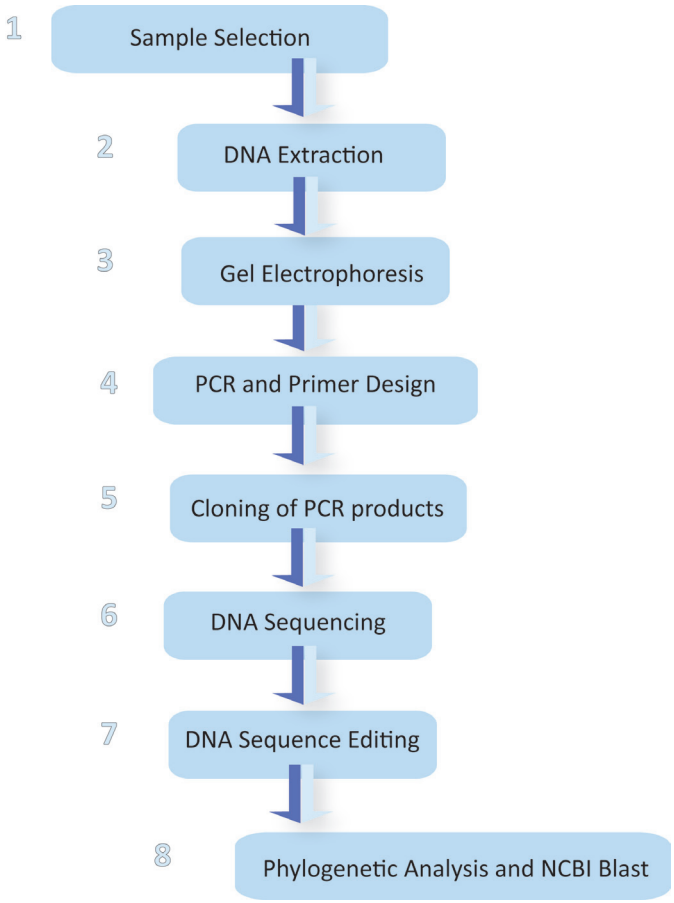

Figure 1. Flow Chart for Part 1.

Table 1. Student outline.

Lab	Experiment	Concepts
Lab 1	Lab safety, notebooks, metric system exercises, and pipetting exercises.	General lab standard
Lab 2	Sample selection and DNA Extraction	Principles of DNA extraction (cell disruption, separation of DNA, precipitation of DNA, and resuspension)
Lab 3	Electrophoresis of gDNA and PCR with universal 18S nrDNA primes	1) Principles of electrophoresis with emphasis on DNA. Polymerase chain reaction (PCR). 2) Primer Design
Lab 4	Electrophoresis of PCR products and gel cleanup of PCR products.	Reinforcement of previous concepts
Lab 5	Cloning PCR products	Plasmids as vectors, restriction endonucleases, transformation (focusing on bacteria).
Lab 6	Screening clones for successful insertion of a PCR product.	DNA sequencing I – traditional methods
Lab 7	Plasmid DNA extraction and sequencing reaction	DNA sequencing II – "next generation sequencing"
Lab 8	Assessment of sequencing – contig assembly	Quality of results from a DNA sequencing reaction. Introduction to basic alignment
Lab 9	Basic blast tools and sequence alignment	Introduction to tools available through NCBI
Lab 10	Phylogenetic analysis I	Basic concepts behind phylogenetic analysis
Lab 11	Phylogenetic analysis II	*Continued.*
Lab 12-14	Extra days typically required for trouble shooting problems with any of the above steps.	

Sample Selection

A wide variety of ecological samples are applicable to the investigation of environmental microbial diversity through nr DNA analyses. Within our local ecosystem, the Chihuahuan Desert, we have had greatest success using samples from moist or aquatic sites. These include ponds, recently flooded arroyos, as well as sludge or water from the Rio Grande. Similar success was found probing for biodiversity in the scat of various local species, detritus in salt and fresh water aquaria, and detritus from growth chambers of various organisms being raised on campus for research purposes. As expected, we had much greater success with amplification of DNA from this range of environments than from drier and/or saltier sites within the region.

DNA Extraction

Samples were subject to DNA extraction using the Qiagen QIAamp DNA Stool Mini Kit (Cat. No. 51504), which is often used for environmental DNA extraction because the manufacturer includes components that reduce the action of PCR inhibitors found in environmental samples. Microbial components in liquid samples were typically concentrated through centrifugation prior to DNA extraction.

Gel Electrophoresis

Following an introduction to the concepts and principles involved in gel electrophoresis, students first applied the technique on their genomic extracts using 0.75% agarose gels stained with sybr safe (Cat. No. S33102). Subsequently, the process of gel preparation and alternations in percent gel and running conditions were elaborated upon in each subsequent step, nearly every one of which required gel electrophoresis to determine the outcome.

PCR and Primer Design

Students were introduced to the basic principles of PCR primers through a discussion of primer design and targeted amplification of gene regions. For this experiment, semi-universal eukaryote-biased 18S rDNA primers were tested from a variety of sources; however, we have had greatest success with those developed by Dawson and Pace (2002). Of the four primer sets published by these authors, the combination of primers 82FE 5'-GAADCTGYGAAYGGCTC-3' with 1492R 5'- CCTTGTTACGRCTT-3' provided the most consistent amplifications in our hands, but also amplified prokaryotic sequences in many samples (see Instructor Notes). The PCR reaction (35.5 µl) for each sample included: 24 µl water, 3.33 µl 10X PCR buffer (this includes 1.5 mM $MgCl_2$), 1.33 µl of dNTPs (from 10 mM dNTP stock), 0.83 µl of Primer F (82FE or 360FE, 20 µM stock), 0.83 µl of Primer R (1492R or 1391RE, 20 µM stock), 1.66 µl of BSA (10 mg/ml stock), and 0.43 µl of Taq polymerase (ca. 2.5U) and 25 ng of environmental DNA (in 3 µl volume). Successful amplifications were typically noted for products around 1500 bp in size.

Cloning of PCR products and DNA sequencing

PCR products were run on 1% agarose gels and the band of interest (ca. 1500 bp) excised to eliminate smaller non-specific products that can complicate subsequent ligation in the cloning step. DNA was recovered from the agarose blocks using either the Qiagen Gel Extraction Protocol (Cat. No. 28704) or a similar protocols available more economically from Epoch Life Science (**http://www.epochlifescience.com/**). Fragments were cloned using the Invitrogen Topo-TA Cloning Kit (Cat. No. K4500-01) and amplicons were recovered from positive colonies using standard plasmid miniprep extractions. Students screened ten colonies for the presence of an appropriately sized insert using PCR and M13 amplification primers and grew five positive colonies for subsequent plasmid isolation and sequencing. Students failing to recover sufficient numbers of transformed cells use extra colonies from other students to complete their set. Lectures and discussions relating to the process of cloning focused on the discovery of transformation in natural systems and the subsequent application and importance of ligations and transformation to modern molecular biology. Forward/reverse sequencing reactions were either carried out by the students and analyzed on campus or, in order to save time, with the University of Chicago DNA sequencing facility (ca. $2.50/sample - **http://cancer-seqbase.uchicago.edu/**). Students were also introduced to modern advances in genome scale sequencing, an experience that was enhanced by NMSUs Roche 454 DNA sequencing facility with which the students were likely to interact with in their future research projects.

DNA Sequence Editing

The informatics component of the experiment took advantage of a combination of commercially and publicly available software packages. The quality and length of each DNA sequencing read as well as the assembly of forward and reverse sequencing reads into contigs were conducted using Seqman in the DNA Star Package (**http://www.dnastar.com/**). DNA Star offer free time-limited licenses of this software package for educational purposes.

Phylogenetics and NCBI BLAST

Following the assessment of DNA sequence quality and the construction of contigs, students were introduced to the data available in international databases (e.g., GenBank) and the utility of BLAST (Altschul *et al*., 1990) searches. BLAST results confirmed whether the sequences were 18S and identified organisms with highly similar sequences that were likely to be close relatives to species in the starting samples. Students further explored the taxonomic affinities of the unknown samples and their relatives through online resources like the Tree of Life Project (**http://tolweb.org/tree/**).

Subsequently participants added all the newly recovered 18S sequences to an 18S dataset that included representatives of the tree of life (e.g., Archaea, Eubacteria, and major clades of Eukaryotes). These sequences were aligned using the software package Muscle through the EMBL-EBI website (**http://www.ebi.ac.uk/Tools/msa/muscle/**). Following an evaluation of alignment quality each student went through phylogenetic analysis using parsimony and maximum likelihood techniques. Parsimony reconstructions were conducted using WinClada and NONA (see **www.cladistics.com** for both) with maximum likelihood run online through RAxML (**http://phylobench.vital-it.ch/raxml-bb/**). File format input options are provided on the associated web pages. While WinClada is a standalone package that can efficiently view and print trees, Treegraph2 (**http://treegraph.bioinfweb.info/**) can be useful for viewing, editing, and printing phylogenetic trees from RAxML. For those less well versed in the interpretation of phylogenetic trees, Baum *et al*. (2005) provide an excellent resource in the "Tree Thinking Challenge" that provides excellent online quizzes for students (**http://www.sciencemag.org/content/310/5750/979.full.pdf**).

Part II: A Reverse Genetics Experiment To Characterize The Function Of Aquaporin Water Transporters In A Mosquito

Introduction

Reverse genetics is a strategy where the researcher uses available DNA sequence data to characterize the phenotype associated with this particular sequence (Lawson and Wolfe, 2011). The opportunities to plan and perform reverse genetic experiments are currently growing exponentially together with the amount of available genomic sequence data for different model and non-model organisms.

The reverse genetics experiment we performed in the second half of the class utilized RNA interference (RNAi), a powerful method to knock down genes in organisms (Shan, 2010). We used it to study the importance of aquaporin water transporters for water excretion in mosquitoes. We chose the Southern House Mosquito *Culex quinquefasciatus* since we have year round access to these mosquito species and, most importantly, its genome sequence has been published recently (Arensburger and al, 2010). Aquaporins are a very appropriate protein family for these experiments because all insects analyzed so far have between six and ten of these genes in their genome and their involvement in diuresis has been clearly demonstrated (Drake et. al, 2010). As an alternative to mosquitoes, any other insect species with available genome sequence could be used for this experiment given that it is not too small and able to survive the injection procedure described below. The American Etymological Society as recently started a 5000 insect genome project that should broaden the choice of accessible model insect species (**http://www.entsoc.org/press-releases/entomologists-launch-5000-insect-genome-project-i5k**). As an alternative to aquaporins, any genes that have a high probability to be involved in insect diuresis could be chosen for example the different subunits of the v-ATPase or several ion exchanger proteins.

Our goal in developing this experiment was to involve students in a real primary research project and lead them through the whole process from the planning phase to the statistical analysis of the acquired data. The students worked together in groups of two. The experiment was divided in eight stages (Fig. 2 and Table 2).

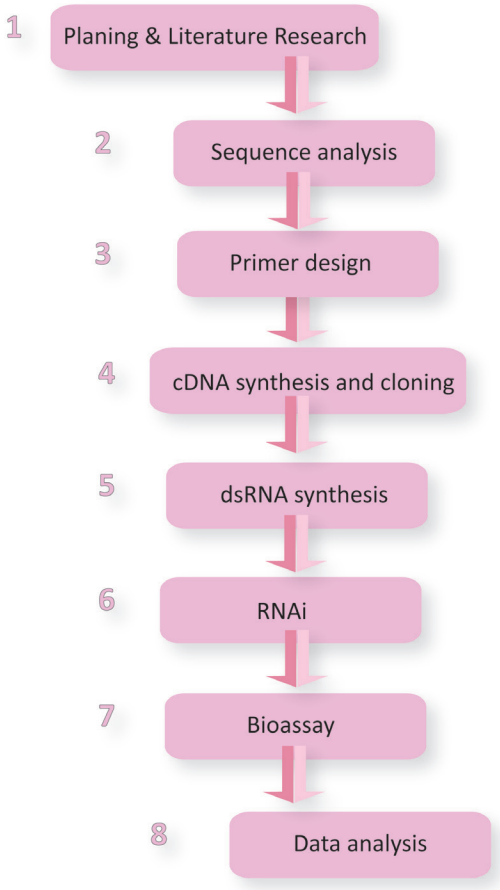

Figure 2. Flow Chart for Part II.

Table 2. Protocol for Part II. Many of the steps were repetitions of the steps followed in Part I, for example, performing DNA minipreps. Therefore students were able to perform the given tasks relatively quickly and accurately.

Lab	Experiments	Concepts
Lab 1	Intro	Performing a reverse genetics experiment
Lab 2	Sequence identification and primer design	DNA/RNA/Protein, structure of genes, genome databases, search tools, Primer-BLAST, primer ordering
Lab 3	RNA isolation from insect tissues	Nucleic acid purification via Tri-reagents
Lab 4	cDNA synthesis	Principles of Reverse Transcription
Lab 5	PCR of cDNA w/ gene specific primers	Reinforcement of previous concepts
Lab 6	Run gels, cut out bands	Reinforcement of previous concepts
Lab 7	gel extract., TOPO reaction and transformation	Principles of TOPO TA cloning, transformation via electroporation, blue white selection
Lab 8	minipreps and digest	
Lab 9	gel, PCR T7 primers	
Lab 10	PCR cleanup, dsRNA	
Lab 11	clean RNA	
Lab12	Injections	RNAi
Lab 13	Diuresis assay	Principles of insect water homeostasis
Lab 14	Statistical analysis	Biometry

In the first stage, we asked the students to do literature research on aquaporins and to focus on what is known about their function in insects. They had to summarize the results of their research in form of a short paper (Appendix).

In the second step the students performed a bioinformatics analysis to identify aquaporin genes in the published genome sequence of *Culex*. They were asked to use the BLAST search tool at www.vectorbase.org. Every group search individually using different BLAST tools (BLAST N, BLAST X, etc.). The results were summarized at the whiteboard at the end of this step. We were able to identify six putative aquaporin genes in the genome of *Culex* .

In the third step students were assigned one specific aquaporin gene and asked to use PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to develop specific primers to amplify a 300 base pair long sequence of their gene's cDNA. PrimerBLAST is based on the Primer3 software but aligns possible primers against the genome to test for specificity. At the end of this step every group submitted the sequence information for a forward and a reverse primer for their gene to the teaching assistant which placed the order immediately. Primers can be ordered from a multitude of commercial companies and usually arrive three to four days after placing the order.

The fourth step included total RNA isolation from mosquitoes using a TriReagent followed by reverse transcription. After the first strand synthesis students amplified partial cDNAs of their assigned genes with the gene-specific primers they developed. The PCR product was analyzed on an agarose gel, purified and cloned into pCRII-TOPO. Successful integration in this plasmid vector was confirmed with restriction digestion followed by agarose gel electrophoresis. Plasmids were sent to a commercial sequencing facility (see above). When the sequencing data became available the students compared their cDNA sequence with the published aquaporin cDNA using ClustalW). Primers for successfully cloned cDNAs were reordered at this time with a T7 sequence added at the 5' end of both, forward and reverse primers (5′ d(TAATACGACTCACTATAGGG) 3′).

In the fifth step, the new primers were used to reamplify the cDNA fragments this time with T7 primer sequences attached on both ends. Template for these PCRs were the pCRII-TOPO plasmids created in step four. Afterwards the students synthesized double stranded RNA against their gene of interest using their PCR product as template and the Megascript T7 Kit (Invitrogen). The dsRNA was purified via LiCl precipitation, pelleted, and dissolved in water to a concentration of 500 ng/µl.

Next, the students constructed a microinjection device that they used to inject 500 ng of dsRNA into individual female *Culex* mosquitoes (see Figs. 3 A & C). Control mosquitoes were injected with dsRNA against a jellyfish protein that has no homologue in insects (GFP). Knockdown mosquitoes were pooled in paper cups and incubated for three days in an insect incubator.

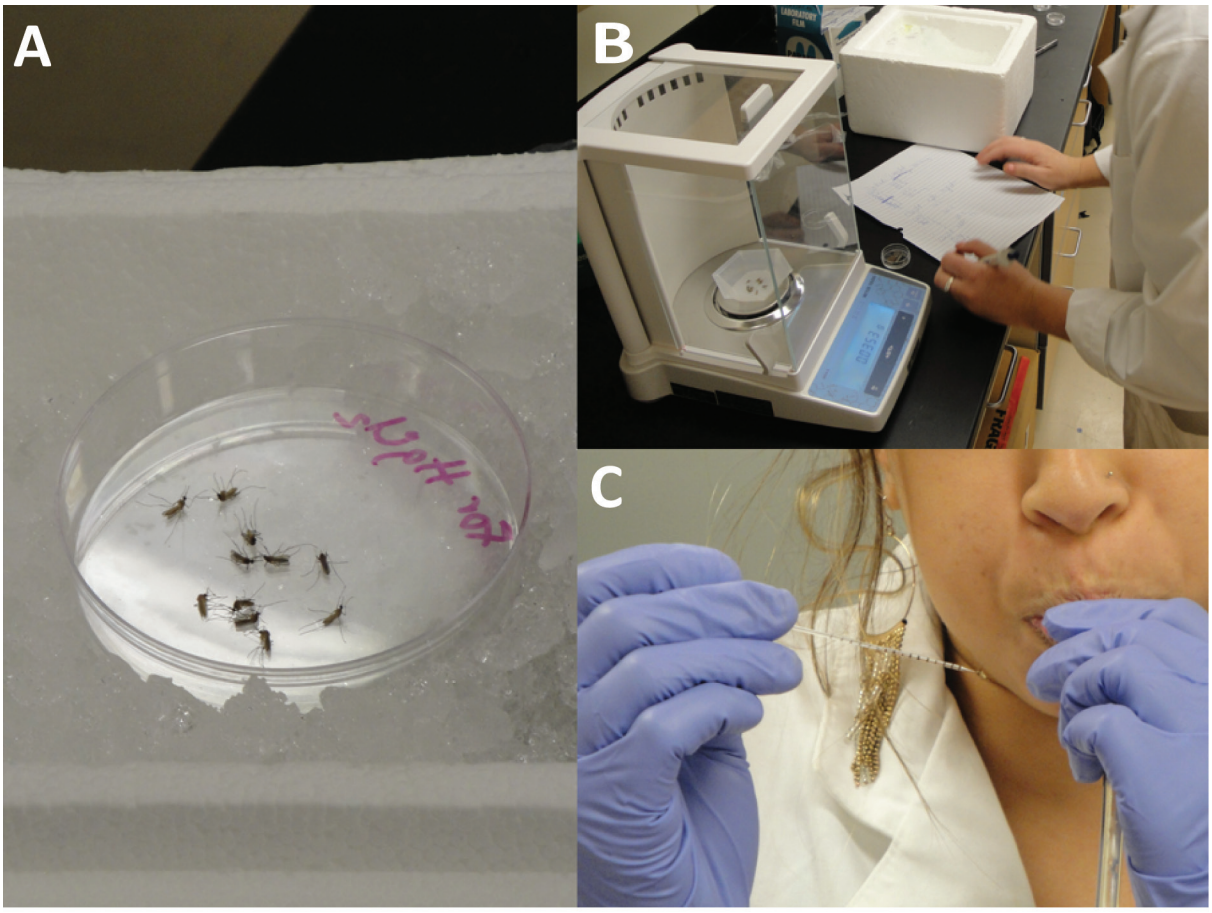

Figure 3. Photos from Spring 2011. A – Mosquitoes can be anaesthetized using ice; B – mosquito weight measurement; C – injection of PBS into mosquitoes with a self made injection device. For information how to construct this device, please contact the authors.

In the seventh step, groups of five knockdown and control mosquitoes were weighed on a precision balance (see Fig. 3B). Afterwards all groups were injected with 1.5 µl PBS (phosphate buffered saline) per mosquito. After injection groups were weighed on a precision balance a second time and kept at room temperature for one hour. Afterwards the group weight was determined again. The diuresis bioassay is published by Drake *et al*. (2010).

In the last step, the volume of injected PBS/group was determined by subtracting the initial weight from the weight taken directly after injection. The excreted volume was calculated by subtracting the final weight from the second weight and excretion rates were determined by dividing the excreted volume by the injected volume. Excretion rates of knockdown and control mosquitoes were compared.

Notes for the Instructor

Part I

We recommend that each environmental sample be replicated with two or three other students to permit sharing of resultant components when one or more students fail to obtain the desired result. Common problems have included limited 18S amplification from samples with low overall DNA concentration. In addition, it has not been uncommon to amplified ca. 50% bacterial 16S DNA even though we have included a "eukaryote specific primer". This is likely due to the relaxed amplification conditions we sometimes resort to for amplification from many samples. This is not a significant problem for the broad purpose here; however, aligning all bacterial and eukaryotic sequences can be difficult in some cases. Note that numerous additional resources are available at:
http://www.scientificsocieties.org/aps/proceedings/soilmicrobes/pages/resources.htm.

Part II

One important challenge in this class was to make sure that every student was provided with materials enabling her/him to perform all eight steps of this experiment. In the last version of this class, we had 18 students working on six aquaporin genes, resulting in 3x coverage for each gene. We strongly recommend this ratio to maximize the chance of complete success in performing this sort of experiment. When, for example, an initial primer pair that a particular student developed (step 3) didn't work as expected the student was provided with another pair from one of his peers that did, or when the cloning step into pCRII-TOPO failed students picked colonies from other students plates that worked on the same gene. With this strategy we were able to maximize the positive experiences that students had during the course of this class.

The data we were able to generate during this course did not have the quality necessary to justify publication. However, the course provided important information about which primers are suitable for analysis of *Culex* aquaporin expression. An undergraduate researcher is currently repeating this experiment to produce publication-ready data.

Acknowledgements

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About the Authors

Immo A. Hansen received his Diploma in Biology and his Doctor Rerum Naturalium (Ph.D.) from the University Würzburg in Germany. He was a postdoc and Assistant Research Entomologist at the Department of Entomology at the University of California Riverside and is now an Assistant Professor at New Mexico State University in Las Cruces, New Mexico. He has taught classes on Evolution and Disease Vector Biology on the Graduate level, introductory classes on Molecular Biology and Cell- and Organismal Biology, as well the Molecular Biology lab described here. His research focuses on the molecular physiology of the yellow fever mosquito Aedes aegypti in particular on water- and amino acid transporter proteins in the mosquito alimentary system.

Angus L. Dawe received his BSc (Hons) degree in Microbiology from Sheffield University in the UK in 1989. He then relocated to Knoxville, Tennessee for graduate school, gaining a MS in Biotechnology (1991) and PhD in Cellular, Molecular and Developmental Biology (1996). Dr. Dawe worked as a postdoctoral fellow at the Robert Wood Johnson Medical School in Piscataway, New Jersey, from 1996 to 1999, before transitioning to the post of Senior Scientist at the University of Maryland Center for Biosystems Research in College Park. Since 2004, Dr. Dawe has been a faculty member in the Department of Biology at New Mexico State University. At NMSU, Dr. Dawe teaches General Microbiology, Molecular and Cellular Mycology, as well the Molecular Biology lab described here. Dr. Dawe's research interests focus on molecular, genetic and genomic studies of filamentous fungi to investigate molecular mechanisms of plant pathogenesis, cellular communication, intracellular signaling pathways and virus-host interactions.

C. Donovan Bailey earned his Bachelors of Science in Botany from UC Davis in 1992 and his Ph.D. in Plant Evolutionary Biology from Cornell University in 2000. His dissertation work focused on the use of molecular tools to investigate phylogenetic relationships among members of the mustard plant family. Following the completion of his Ph.D., Dr. Bailey accepted a postdoctoral position in the Department of Plant Sciences with the University of Oxford (UK) where he studied the evolution of semi-domesticated crop species from central Mexico. In 2003 he joined the Biology faculty at NMSU where he continues to focus his research on plant evolutionary biology and enjoys instructing a range of courses including Introductory Biology, Plant Evolution, and Molecular Biology.

Appendix A: Lab Report

BIOL 302 Take home assignment: *Culex* Aquaporin

Task

Write a short review (not more than three pages, four with citations) with focus on *Culex pipiens quinquefasciatus* and what is known about aquaporins and specifically mosquito aquaporins (this assignment is worth 50 pts).

In your review address the following topics:

- *Culex* as vector of disease
- Aquaporin protein structure
- Aquaporin transport mechanism
- Regulation of water transport activity
- Aquaporin function in mosquitoes

Please cite at least ***five*** references in JBC style, one on the role of C. quinquefasciatus as disease vector and four on aquaporins:

- JBC stands for "Journal of Biological Chemistry"
- You have to go online and find out what JBC style is by checking the 'instructions for authors' (or by looking at an actual JBC paper.

Format: Times New Roman, Font size 12, line spacing Double

Due date: April 7th, 2011, **please submit a hard copy of your review.**

A good website to start your literature research is:
http://www.ncbi.nlm.nih.gov/pubmed/

Keywords:

- _____
- _____
- _____

The usual sanctions apply for plagiarism: 0 points!

Appendix B: Equipment & Supplies (US Dollars)

	Annual Expenditures based on a 10 year life expectancy	Price/ Student (18 students / year)
Equipment		
Thermocycler	1400	77.78
Gel documentation	800	44.44
Gel electrophoresis	800	44.44
Pipetters	720	40.00
UV transilluminator	175	9.72
Fridge/freezers	80	4.44
Centrifuges	40	2.22
tube racks	40	2.22
Incubators	20	1.11
Waterbath	20	1.11
Thermoprinter	50	2.78
Equipment total	**$4145**	**$230.28**
Supplies		
Pipetter tips	1000	55.56
Invitrogen Kits	920	51.11
Gloves	904	50.22
Qiagen Kits	554	30.78
Primers	450	25.00
Ambion Kits	265	14.72
15 ml tubes	250	13.89
XGAL	216	12.00
Taq Master Mix	173	9.61
Trizol reagent	151	8.39
Petri dish	150	8.33
PCR tubes	100	5.56
Glassware	100	5.56
Eppendorf tubes	90	5.00
LB agar	88	4.89
Thermoprinter paper	77	4.28
Chloroform	71	3.94
LB medium	59	3.28
Antibiotics	50	2.78
IPTG	50	2.78
Isopropanol	45	2.50
Ethanol	37	2.06
Methanol	30	1.67
Supplies total	**$3828**	**$323.89**
Annual Expenditure/Student	**--**	**$554.17**

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