

ARTICLES

METHODS FOR SEQUENCING THE MITOCHONDRIAL DNA A+T-RICH REGION OF *COCHLIOMYIA MACELLARIA* (DIPTERA: CALLIPHORIDAE) FROM NORTH AMERICA

PETRA BOEHME¹ AND JEFFREY D. WELLS²

ABSTRACT

The A+T-rich region (ATRR) is the major non-coding segment of insect mitochondrial DNA (mtDNA). It is a hypervariable locus, and therefore useful for population genetic studies. Forensic entomologists could potentially use the ATRR to test for regional subpopulations of a carrion insect species, or to reconstruct the post-mortem movement of a corpse. However, the ATRR presents greater genotyping difficulties compared to the commonly studied mtDNA coding regions. Homomeric stretches and length heteroplasmy within the ATRR, and a lack of conserved internal PCR primers, so that PCR amplicons of around 1500 bp are necessary, make both direct sequencing and sequencing of cloned product difficult. We designed ATRR internal primers, complementary to the same annealing site, and tested them using *C. macellaria* specimens from Puerto Rico, Florida, Tennessee, and West Virginia. The entire ATRR sequence, other than the primer site, could be obtained from cloned PCR product. The ability to use shorter PCR amplicons should also make this method more suitable for analyzing degraded evidence.

RÉSUMÉ

Une région AT-rich (ATRR) est un segment majeur de non-codification d'insecte mitochondrial ADN (mtDNA). Une région est un lieu géométrique hypervariable et utile pour les études génétiques d'une population. Les entomologistes légaux pourraient potentiellement utiliser l'ATRR pour évaluer les sous-populations régionales d'une espèce d'insecte charogne, ou reconstruire le mouvement rétrospectif d'un cadavre. Néanmoins, l'ATRR présente de plus grandes difficultés génotype comparativement au mtDNA codage régional qui est généralement étudié. Les étendues homomeric, les longueurs heteroplasmy dans l'ATRR et le manque d'abécédaires PCR intérieurs conservés pour qu'un PCR amplicons d'environ 1500 bp soient nécessaires, rend les séquences directes et les séquences de produit de clonage difficile. Nous avons conçu des abécédaires intérieurs ATRR complémentaires au même site recuisant et les avons évalués utilisant *C. macellaria* les exemplaires de Porto Rico, la Floride, le Tennessee et la Virginie Occidentale. La séquence ATRR en entier, autre que le site d'abécédaire de base, pourrait être obtenu du produit PCR cloné. La capacité d'utiliser de plus petits PCR amplicons devrait aussi rendre cette méthode plus convenable pour l'analyse des évidences dégenérées.

1. Institute of Legal Medicine, Johann Wolfgang Goethe-University of Frankfurt, Kennedyallee 104, D-60596 Frankfurt am Main, Germany.
2. Department of Biology, West Virginia University, Morgantown, WV 26506, USA.

INTRODUCTION

The secondary screwworm *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) occurs from Argentina to Canada (1), and is one of the more common blow fly species used as evidence in a death investigation involving decomposing human remains (2). As with many other forensically important insects, scientists have developed *C. macellaria* molecular genetic data for purposes of systematics (3) and specimen identification (e.g. 4, 5). More recently attention has been focused on the development of hypervariable genetic data for blow flies, i.e. those that can be used to investigate population genetic phenomena (6-8).

Population genetic studies could contribute to forensic entomology in several ways (9). For example, if genetically distinct regional populations were detected, we believe that basic biological information, such as development rate, should be gathered separately for each of the populations. Maggot kinship analysis might allow an investigator to reconstruct the post-mortem movement of a corpse. This is because maggots can be left behind at the original site after a corpse is moved (10). If a maggot found at one location were shown to be a sibling of a maggot of similar age infesting a corpse at a different location, this would strongly suggest that the victim's body had been moved between the two sites.

Although a nuclear locus, such as a microsatellite, is often preferred for population genetic studies (9), mitochondrial DNA (mtDNA) may be the only material available for a degraded specimen (11), such as is often the case for forensic evidence. The most concentrated occurrence of polymorphic base positions within insect mtDNA is likely to be within the non-coding A+T-rich region (ATRR, 12-14). Therefore this locus could be useful for population studies of forensically important groups such as the Calliphoridae, as has been shown to be the case with *Drosophila subobscura* (Diptera: Drosophilidae) (15). As the name implies, this region is rich in adenine and thymine nucleotides, and the reported size ranges from 350 bp in Lepidoptera (16), to 4600 bp in *D. melanogaster* (17).

The ATRR in *C. macellaria* was described by Lessinger and Azeredo-Espin (18). It is about 1220 bp long, and is flanked by the genes for 12S rRNA and tRNA isoleucine. Domain A, located near the tRNA isoleucine gene, included eight sequence blocks that are highly conserved between species. Domain B, close to the 12S rRNA gene, is more variable.

The ATRR contains a variety of repeat regions (19, 20). These result in length heteroplasmy, which impedes direct sequencing. Flanking gene duplication has also been observed (21), and this will cause problems if a primer site is duplicated. Current methods for amplifying the *C. macellaria* ATRR yield PCR product more than 1500 bp in length (18). Even if the problem of length heteroplasmy is avoided by cloning PCR product, such PCR amplicons are too long to completely sequence using standard technology.

Primers that anneal within the ATRR would produce shorter fragments that would be easier to sequence, and recently two ATRR internal primers were successfully designed for *Chrysomya* spp. (21). However, those primers did not work for our *C. macellaria* specimens. This study was an attempt to design and test new internal primers that can be used to get additional ATRR sequence data for forensically important North American flies (22), beginning with *C. macellaria*.

MATERIAL AND METHODS

Specimens

A single *C. macellaria* adult originating from each of the following locations was used: Lago Rojo, Puerto Rico (PR); Gainesville, Florida (FL); near Great Smoky Mountains

National Park, Tennessee (TN); and Morgantown, West Virginia (WV). Most were wild-caught specimens, except for FL, which was from a laboratory colony. Flies were killed and stored in 95% ethanol until DNA extraction. Specimens were identified using the keys of Dear. The remains of each specimen were deposited as a voucher in the West Virginia University Entomological Collection.

DNA extraction and Polymerase Chain Reaction

Genomic DNA was extracted from the thoracic muscle (PR, FL) or six legs (WV, TN) using the QIAgen Dneasy® Tissue Kit (Valencia, CA) and following manufacturer's instructions. The final elution volume was 200 µL.

Amplification was performed in a total volume of 25 µL with a Promega master mix (Madison, WI) containing 5 pMol of each primer and 1 µL of the DNA extracts as a template. The thermal cycler program was 94°C for 30 s, 58°C for 1 min and 60°C for 2 min for 30 cycles followed by a final extension at 60°C for 10 min (18).

All PCR reactions were performed in a GeneAmp PCR System 9700 Thermal cycler (Applied Biosystems, Foster City, CA), PCR products were separated by gel-electrophoresis in a 1% agarose gel, and DNA was visualized with SYBR Green under UV light.

Primers

Two new internal primers, AT-J-15660 (5'-CCATTTTTATATTGAAGCGTGC-3') and AT-N-15639 (5'-GCACGCTTCAATATAAAAATGG-3'), were designed based on the published ATRR sequence for a Brazilian *C. macellaria* specimen (GenBank accession AF151385; (18)). These primers are reverse complements of each other, i.e. they anneal at the same location within the ATRR. The names follow the common convention (23), with the number indicating mtDNA base position in the system established by Clary and Wolstenholme (24) for *Drosophila yakuba*. They were used in combination with primers that anneal in the 12S rRNA gene (SR-J-14776, 5'-GCTGGCACGAATTTTGTC-3', (18)) and the tRNA methionine gene (TM-N-193, 5'-TGGGGTATGAACCCAGTAGC-3', (16)).

Amplification of the ATRR

Initial amplification of a ca. 1580 bp region including the entire ATRR was performed using the primer pair SR-J-14776 and TM-N-193. One µL of this long target served as template for nested reactions with primers SR-J-14776 and AT-N-15639, or TM-N-193 and AT-J-15660, producing amplicons of ca. 900 and 700 bp, respectively.

Cloning and Sequencing

Long target and nested PCR products were cleaned using the QIAquick Purification Kit® (Valencia, CA) following manufacturer's instructions. Purified products were cloned into a vector with the Invitrogen, TOPO TA Cloning Kit® (Carlsbad, CA) using one-half the recommended reagent volumes with one-fourth of competent cells. Five clones of each PCR product were selected and the plasmids purified with the Invitrogen S.N.A.P.™ MiniPrep Kit following the recommended steps.

Cleaned plasmid DNA was either sequenced by the commercial service Macrogen (www.macrogen.com), or with BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA). Sequence data were obtained for forward and reverse DNA strands, and data files were edited and aligned using Sequence Navigator software (Applied Biosystems, Foster City, CA).

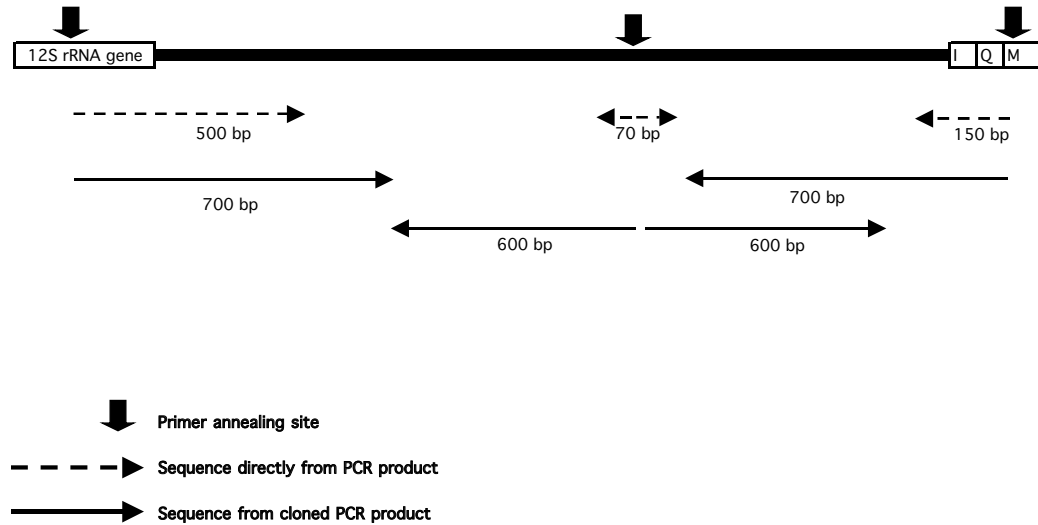


Figure 2. Diagram of the *Cochliomyia macellaria* ATRR and flanking rRNA and tRNA genes, showing the areas that could be sequenced using various protocols.

Direct sequencing

One or two μL of the long target and nested PCR products were sequenced directly (without cloning) using BigDye® Terminator v3.1. A total sequencing reaction volume of 10 μL consisted of 2 μL 5x buffer, either 4.65 or 5.65 μL water, 0.75 μL Big Dye and 0.6 μL primer at a concentration of 5 pMol/ μL . The thermalcycler program was an initial denaturation of 95°C for 2 min, followed by 25 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Clones were sequenced followed the same protocol, except that 3 μL DNA was used as a template.

Sequencing product was purified using spin columns filled with Sephadex beads (Fisher, Pittsburgh, PA). The sequence was detected using an Applied Biosystems 3130 genetic analyzer.

RESULTS AND DISCUSSION

Using all four primers, and cloned PCR product as a template, it was possible to obtain the entire ATRR sequence other than that of the internal primer annealing site (Figures 1 and 2). Certainly the annealing site must very closely match the primers for them to have worked. Complete ATRR haplotypes were deposited in GenBank (PR = EU106650, EU106651; FL = EU106652; TN = EU106653, EU106654, EU106655, EU106656; WW = EU106657).

The direct sequencing reactions failed at repeat regions. Elements, such as long thymine nucleotide stretches (25) cause polymerase slippage and length heteroplasmy (20). Downstream from a length heteroplasmy site, a sequence electropherogram quickly becomes unreadable.

Although we observed intraspecific variation in the form of slightly different clones from the same individual, these preliminary data suggest that it is still possible to use a single haplotype from an individual for interspecific comparisons. The haplotypes from an individual form a closely related lineage (Figure 3), therefore the selection of ATRR haplotype should not affect the results of a phylogenetic or population genetic analysis.

kindly supplied the French abstract. These results were presented at the 2006 Annual Meeting of the North American Forensic Entomology Association, in West Lafayette, IN.

REFERENCES

1. Hall D.G. Blowflies of North America. Thomas Say Foundation Vol. IV, 1947.
2. Byrd J.H. and Castner J.L. Insects of Forensic Importance. In: Byrd J.H. and Castner J.L., Editors, Forensic Entomology. The utility of arthropods in legal investigations, Boca Raton, FL: CRC Press, 2001; 43–48.
3. Taylor D.B., Szalanski A.L., Peterson II R.D. Mitochondrial DNA variation in screwworm. Med. Vet. Entomol. 1996; 10: 161–169.
4. Wells J.D. and Sperling F.A.H. DNA- based identification of forensically important Chrysomyinae (Diptera: Calliphoridae). Forensic Sci. Int. 2001; 120: 110–115.
5. Litjens P., Lessinger A.C., and Azeredo-Espin A.M.L. Characterization of the screwworm flies *Cochliomyia hominivorax* and *Cochliomyia macellaria* by PCR-RFLP of mitochondrial DNA. Med. Vet. Entomol. 2001; 15: 183–188.
6. Florin A.-B. and Gyllenstrand N. Isolation and characterization of polymorphic microsatellite markers in the blowflies *Lucilia illustris* and *Lucilia sericata*. Mol. Ecol. Notes. 2002; 2: 113–116.
7. Torres T.T., Brondani R.P.V., Garcia E., and Azeredo-Espin A.M.L. Isolation and characterization of microsatellite markers in the new world screw-worm *Cochliomyia hominivorax* (Diptera: Calliphoridae). Mol. Ecol. Notes. 2004; 4: 182–184.
8. Torres T.T. and Azeredo-Espin A.M.L. Development of new polymorphic microsatellite markers for the New World screw-worm *Cochliomyia hominivorax* (Diptera: Calliphoridae). Mol. Ecol. Notes. 2005; 5: 815–817.
9. Wells J.D., Stevens J.R. Applications of DNA-based methods in forensic entomology. Annu. Rev. Entomol. In press.
10. Wells J.D., Introna F.G., Di Vella G., Campobasso C.P., Hayes J., and Sperling F.A.H. Human and insect mitochondrial DNA analysis from maggots. J. Forensic Sci. 2001; 46: 685–687.
11. Benecke M. and Wells J.D. DNA techniques for forensic entomology. In: Byrd J.H. and Castner J.L., Editors, Forensic Entomology. The utility of arthropods in legal investigations, Boca Raton, FL: CRC Press, 2001; 346.
12. Fauron C.M.-R. and Wolstenholme D.R. Structural heterogeneity of mitochondrial DNA molecules within the genus *Drosophila*. Proc. Natl. Acad. Sci. USA. 1976; 73: 3623–3627.
13. Fauron C.M.-R. and Wolstenholme D.R. Extensive diversity among *Drosophila* species with respect to nucleotide sequences within the adenine + thymine-rich region of mitochondrial DNA molecules. Nuc. Acids Res. 1980; 8: 2439–2452.
14. Fauron C.M.-R. and Wolstenholme D.R. Intraspecific diversity of nucleotide sequences within the adenine + thymine-rich region of mitochondrial DNA molecules of *Drosophila mauritiana*, *Drosophila melanogaster* and *Drosophila simulans*. Nuc. Acids Res. 1980; 8: 5391–5410.
15. Brehm A., Harris D.J., Hernández M., Perez J.A., Larruga J.M., Pinto F.M., and González A.M. Phylogeography of *Drosophila subobscura* from north Atlantic islands inferred from mtDNA A+T rich region sequences. Mol. Phylogenet. Evol. 2004; 30: 829–834.
16. Taylor M.F.J., McKechnie S.W., Pierce N., and Kreitman M. The Lepidopteran mitochondrial control region: structure and evolution. Mol. Biol. Evol. 1993; 10: 1259–1272.
17. Lewis D.L., Farr C.L., Farquhar A.L., and Kaguni L.S. Sequence, organization, and evolution of the A+T region of *Drosophila melanogaster* mitochondrial DNA. Mol. Biol. Evol. 1994; 11: 523–538.
18. Lessinger A.C. and Azeredo-Espin A.M.L. Evolution and structural organisation of mitochondrial DNA control region of myiasis-causing flies. Med. Vet. Entomol. 2000; 14: 71–80.
19. Moritz C., Dowling T.E., and Brown W.M. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Annu. Rev. Ecol. Syst. 1987; 18: 269–92.

20. Zhang D.-X. and Hewitt G.M. Insect mitochondrial control region: a review of its structure, evolution and usefulness in evolutionary studies. *Biochem. Syst. Ecol.* 1997; 25: 99–120.
21. Lessinger A.C., Junqueira A.C.M., Conte F.F., and Azeredo-Espin A.M.L. Analysis of a conserved duplicated tRNA gene in the mitochondrial genome of blowflies. *Gene*. 2004; 339: 1–6.
22. Boehme P. Population genetics of forensically important North American blow flies (Diptera: Calliphoridae) using the A+T-rich region of mitochondrial DNA. Diploma Thesis, Universität Bonn, 2006.
23. Simon C., Frati F., Beckenbach A., Crespi B., Liu H., and Flook P. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* 1994; 87: 651–701.
24. Clary D.O. and Wolstenholme D.R. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and the genetic code. *J. Mol. Evol.* 1985; 22: 252–271.
25. Saito S., Tamura K., and Aotsuka T. Replication origin of mitochondrial DNA in insects. *Genetics*. 2005; 171: 1695–1705.
26. Swofford D.L. *0PAUP, Phylogenetic Analysis Using Parsimony (and other methods)*. Version 4. Sinauer, Sunderland, MA, 1984.