# USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS AS A NEW METHOD FOR SPECIES IDENTIFICATION IN *DIORHABDA* SPP.


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## Abstract


*Diorhabda* species (tamarisk leaf beetles) were introduced into the United States as a biological control agent for the invasive tamarisk species, with some populations successfully controlling the spread of tamarisk and others failing. It has been proposed that this disparity in efficacy of different populations could be tied directly to the species of each population. The identification of *Diorhabda* spp. through traditional methods is taxing on researcher time, budgets, and laboratory materials. This research has developed a new method for performing species identification of *Diorhabda* spp. that can be performed cheaper, faster, entirely in-house, and with less of an environmental impact than the traditional protocol.

Species identification is traditionally performed by Sanger sequencing of the mitochondrial gene, cytochrome c oxidase subunit 1 (COI), which costs $17.76 per sample and takes 10-14 days to accomplish. This research has developed a protocol that utilizes restriction fragment length polymorphism (RFLP) to target nucleotide sequences within the COI gene that are unique to each species. By performing two restriction enzyme digests that target these non-conserved sites, a significant difference in DNA fragment lengths can be observed through gel electrophoresis that allows for positive species identification. This new protocol can be performed in two days, and at a 72% reduction in cost.


## Materials and Methods

Several CO1 gene sequences of each *Diorhabda* species were collected from GenBank, and a 1300 base pair fragment was analyzed in MEGA7 to determine where each species had unique nucleotide sequences that could be targeted by restriction enzymes. Once a list of all possible sites had been compiled, it was then narrowed by determining which of those sequences had restriction enzymes available via New England Biolabs Enzyme Finder. With the list further narrowed, analysis of the theoretical fragment lengths was started. This involved finding which restriction enzymes had the fewest number of unintended cut sites, determining what the resultant fragment lengths of each species would be with digests involving various combinations of restriction enzymes, and insuring that each species would have enough variation in fragment lengths to uniquely distinguish them from the each other after gel electrophoresis. After compiling a theoretical model that utilized the New England Biolabs restriction enzymes BfaI, AclI, and SwaI, tests were run with previously isolated and sequenced DNA samples to confirm the new protocol's success.

**Digest A (BfaI and AclI)**

| Enzyme | Species | Cut Site |
|---|---|---|
| BfaI | *elongata* | 221 |
| | *sublineata* | 770 |
| AclI | *sublineata* | 1017 |

**Theoretical Gel, Digest A, (BfaI/AclI)**

| *elongata* | *sublineata* | *carinulata* | *carinata* | Ladder |
|---|---|---|---|---|
| | | ■ | ■ | 1300 |
| | | | | |
| | | | | 1200 |
| | | | | |
| ■ | | | | 1100 |
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| | | | | 400 |
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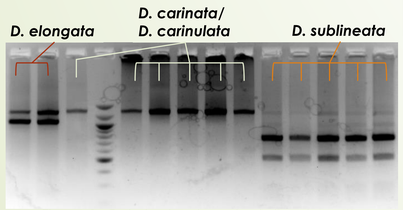


Figure 1: Gel electrophoresis of digest A with BfaI and AclI restriction enzymes.

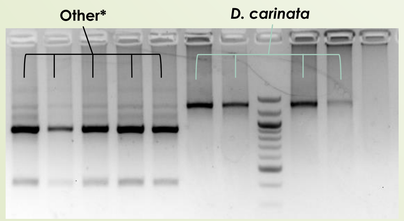


Figure 2: Digest B with SwaI restriction enzyme. *Other species, as identified from Digest A

**Digest B (SwaI)**

| Enzyme | Species | Cut Site |
|---|---|---|
| SwaI | *carinulata/elongata/ sublineata* | 391 |

**Theoretical Gel, Digest B, (SwaI)**

| *elongata* | *sublineata* | *carinulata* | *carinata* | Ladder |
|---|---|---|---|---|
| | | | ■ | 1300 |
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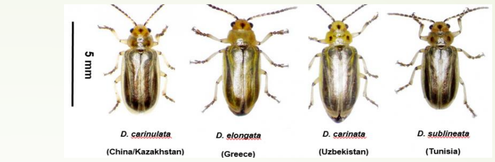


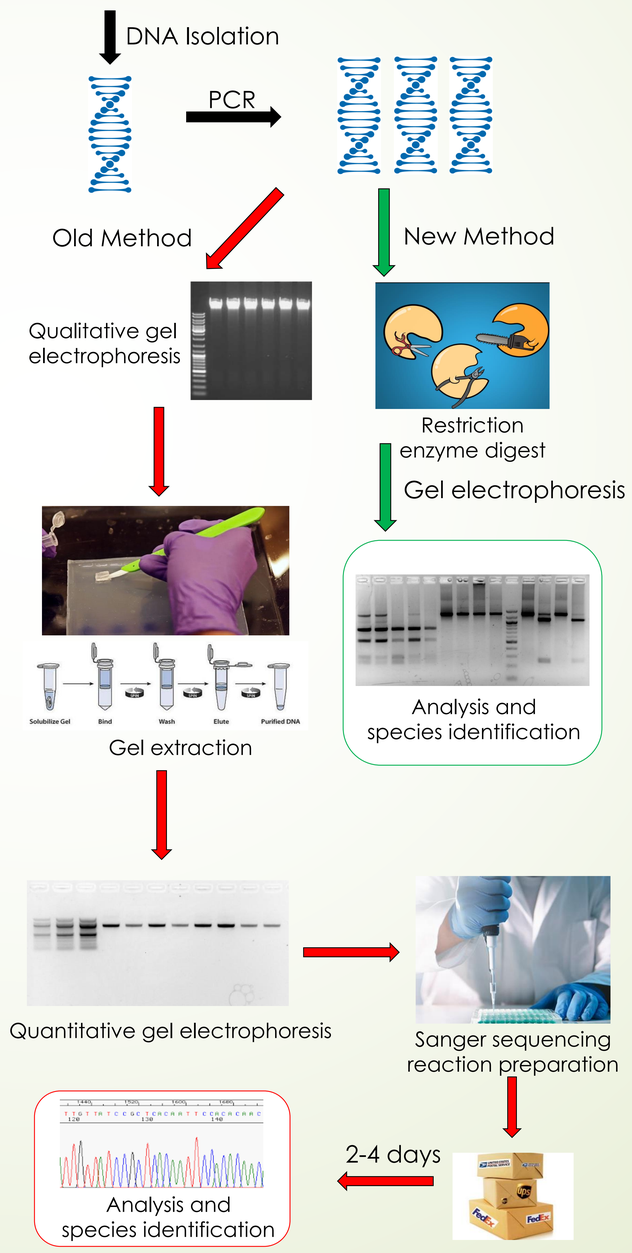


Figure 3: Typical workflow of traditional species identification (Old Method) compared to RFLP protocol (New Method).

## Results and Conclusions

- The new protocol was largely successful, with all 55 initially tested samples responding as predicted.
- The costs of the new protocol are $12.80 less per sample than the traditional method of sanger sequencing. This amounts to a 72.1% reduction in cost per sample analyzed.

## Future Goals and Research

- The protocol will be altered to use restriction enzymes from Thermo Fisher Scientific that will allow the digest to be performed in a single, combined step.
- The new protocol is being implemented as the new standard for maternal *Diorhabda* species identification in our research into convergence of the four species.

| Traditional Sequencing Protocol | | RFLP Protocol | |
|---|---|---|---|
| DNA Extraction/isolation | $2.89 | DNA Extraction/isolation | $2.89 |
| Agarose | $0.60 | Agarose | $0.60 |
| Gel Extraction | $2.27 | Restriction Enzymes | $1.47 |
| Sanger Sequencing | $12.00 | | |
| **Total cost per sample** | **$17.76** | | **$4.96** |

Figure 4: Cost analysis of traditional sequencing protocol versus RFLP protocol on a per sample basis.

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