PHYLOGENETIC REVISION OF DESERT FIREFLIES (COLEOPTERA: LAMPYRIDAE: *Microphotus*)

A Thesis

by

JESSICA LEANN USENER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

December 2004

Major Subject: Entomology

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Approved as to style and content by:

Anthony I. Cognato (Chair of Committee)	Rodney L. Honeycutt (Member)
James B. Woolley (Member)	Kevin M. Heinz (Head of Department)

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ABSTRACT

Phylogenetic Revision of Desert Fireflies (Coleoptera: Lampyridae: *Microphotus*). (December 2004)

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Chair of Advisory Committee: Dr. Anthony I. Cognato

Morphological, mtCOI DNA, and luciferase DNA data are analyzed individually and simultaneously for phylogenetic signal. Analysis of 16 characters traditionally used in species identification for 317 individual *Microphotus* specimens yields 5000 trees with poor resolution. Although mtCOI and luciferase data conflict in basal clades, both contribute to the phylogeny of *Microphotus*. Based on lack of morphological variation and geographic and temporal proximity of collection localities, *M. decarthrus* Fall 1912 and *M. fragilis* Oliver 1912 are synonomized.

Microphotus octarthrus Fall occurs throughout the southwestern United States in discontinuous pinyon-juniper and juniper-oak habitats. Wide geographic distribution, discontinuous habitat and limited dispersal capabilities of females makes this species ideal for the study of genetic variation. Mantel's approximate *t* test indicates that populations are both geographically and genetically isolated. Twenty-six haplotypes are found among 28 individuals; haplotypes are unique for the populations studied. When subjected to a 2.3% sequence divergence rate, mean branch lengths suggest segregation of populations began in the Holocene, before Pleistocene glaciation. Although these

data suggest greater species diversity, more data, including mating behavior and more genes are required to further elucidate species limits.

ACKNOWLEDGEMENTS

Several people were instrumental in the completion of this study. I wish to thank Ed Riley, Joe Cicero, Katherine Stanger-Hall, Mark Branham, and Jim Lloyd for sharing specimens and guidance.

To my major professor, Anthony Cognato, thank you for your guidance and attention to my education and this study. Thank you for your willingness to help in both the field and lab, and thank you for your patience. You have truly made this an enriching and enjoyable life experience. Thank you Drs. Jim Woolley and Rodney Honeycutt for your guidance and constructive criticisms throughout this process.

Thanks also to Mom, Walter, Travis and Beavis who were always there to offer support and take the edge off the stress.

Partial funding for this study was provided by the American Museum of Natural History Theodore Roosevelt Memorial Fund and the Entomology Graduate Student Organization.

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CHAPTER I

INTRODUCTION

Fireflies are probably the most familiar sights on North American summer nights. Approximately 85 genera and 200 species of lampyrids occur worldwide except in Antarctica (Lloyd 2002). About one half of these species occur in tropical America and approximately two to three times as many fireflies are yet to be described (Lloyd 2002). The morphology is mostly canalized and varies allometrically. Thus, within North America there are potentially cryptic species awaiting description. Lampyrids are soft-bodied elongate or elongate-oval shaped beetles, approximately five to twenty millimeters long, and many are capable of emitting light (Arnett 1963, Lloyd 2002).

Three major ecological and behavioral types of lampyrids occur in North America (Lloyd 2002). East of the Rocky Mountains the most commonly recognized type is the dialog or flashing firefly (Lloyd 2002). Males in this group are fully winged and possess flash organs that commonly appear as two pale ventrites on the posterior abdomen (Lloyd 2002). Females in this group may be fully winged, brachypterous, or apterous and may or may not possess flash organs (Lloyd 2002). Flash patterns are used in sexual communication and are often species specific (Lloyd 2002). In the diurnal or day active type both males and females are fully winged and do not possess any light organs (Lloyd 2002). Sexual communication in this group is presumed to occur through the use of pheromones (Lloyd 2002). The third type of firefly is the nocturnal

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This thesis follows the style and format of *Insect Systematics and Evolution*.

glowworm type (Lloyd 2002).Males in this group are fully winged and may or may not possess light organs. They are generally capable of only weak bioluminescence, and do not use their photic organs in sexual communication. Males in this group often have huge eyes that touch or nearly touch under the head (Lloyd 2002). Females are either brachypterous, apterous, or "larviform" and produce intermittent glows rather than flashes to attract males (Lloyd 2002). The females and larvae of this group are presumed to live in burrows or be inquilines of ants (Lloyd 2002). This type of firefly and the diurnal type are most commonly encountered in the western United States (Lloyd 2002). Perhaps due to their poor representation in North America and sometimes unusual habitats, this type has been largely overlooked by contemporary lampyrid workers. As a result, the biology, behavior, and taxonomic relationship of these fascinating creatures are poorly known.

CHAPTER II

SPECIES DIAGNOSIS AND PHYLOGENY OF *Microphotus* USING MORPHOLOGICAL AND MOLECULAR DATA

Microphotus is a small genus of nocturnal glowworm fireflies occurring throughout the southwestern United States and adjacent parts of Mexico. Males are recognized by the following characteristics (Green 1959, LeConte 1866): 1.)The large, prominent eyes touch or nearly so under the head; 2.) antennae are shorter than the pronotum and composed of eight to ten antennomeres with a small, glassy, bead-like process on the tip of the terminal segment; 3.) elytra shorter than the abdomen and usually pale colored with darker tips; and 4.) mouthparts reduced and barely visible between the eyes. All males possess a medial triangular or lobate process on the penultimate abdominal ventrite. Females are apterous and larva-like in appearance. Although originally described otherwise, both males and females possess paired photic organs on the terminal abdominal ventrites. Females emit intermittent glows to attract males. Males, while capable of emitting weak glows or flickers, apparently do not use bioluminescence in sexual communication.

Few taxonomic treatments of the genus have been conducted. J. L. LeConte (1866) first described *Microphotus*. Prior to 1959, eight species and one subspecies were described by LeConte (1866, 1874), Fall (1912), and E. Oliver (1911, 1912). J. W. Green (1959) revised the genus, adding a new name (*M. chiricauhuae*), synonomizing three names (*M. robustus, M. rinconsis*, and *M.abbreviatus*), and elevating the only

subspecies (*M. octarthrus pecosensis*) to species status. Currently, seven species names are recognized.

Few diagnostic morphological characters separate the species. In his revision, Green (1959) relied primarily on male genitalia and secondarily on elytral length, color and number of anntenomeres to define species limits. Relatively few specimens representing few disjunct populations were dissected or examined. Cursory observation of additional specimens suggests the taxonomic characters may not diagnosis the species.

Wheeler and Platnick (2000) define a species based on a unique combination of characters shared among all individuals. In this definition, phylogenetic characters are attributes found in all individuals of a terminal lineage, whereas traits are attributes that are not universally distributed among comparable individuals in a terminal lineage. (Nixon & Wheeler 1990). One cannot know prior to phylogenetic analysis whether supposed characters are in fact either phylogenetic characters or traits. In some cases, variation among individuals is either ignored or concatenated for an OTU that may or may not represent a species. This results in an artificial suite of characters not necessarily observed in any real organism (Vrana &Wheeler 1992). The use of individuals as terminals tests the phylogenetic utility of suspected characters especially those that are polymorphic (Vrana & Wheeler 1992). Poor resolution from a phylogenetic analysis indicates a failure to reject the hypothesis, thus suggesting that suspected characters are likely traits. Rejection of the hypothesis would indicate that the

morphological differences are indeed characters and monophyly of individuals provides evidence of species limits (Wheeler &Platnick 2000).

This study examines the characters used by Green to define species limits at the level of population in order to assess the phylogenetic utility of these characters. In addition, DNA sequences from the mitochondrial cytochrome oxidase I gene and the nuclear luciferase gene are examined for their utility in defining species limits within *Microphotus*. These genes were chosen because of their potential to reveal phylogenetic information at different taxonomic levels (Graybeal 1994). Cytochrome oxidase I has been used extensively for beetles, especially when examining closely related species and populations (Caterino et. al. 2000) and represents a neutral marker. The evolution of the luciferase genome may be under sexual selection because of its intimate association with sexual communication. Luciferase is the only enzyme interacting with the substrate luciferin to create the bioluminescent sexual signal utilized by many firefly species. Variation in mating behavior in part is likely to arise in response to substitutions in luciferase (Kim et al. 2004). Therefore, variation in luciferase may reflect variation in species specific mating behavior. Phylogenetic analysis of these characters should reveal distinct species boundaries represented as monophyletic groups.

MATERIALS AND METHODS

Adult *Microphotus* specimens were obtained on loan from the following institutions and individuals.

CAS: Department of Entomology, California Academy of Sciences, San Francisco, CA (David Kavanaugh, Roberta Brett)

CDFA: California State Collection of Arthropods, Plant Pest Diagnostics Branch, California Department of Food and Agriculture, Sacramento, CA (Chuck Bellamy)

CU: Cornell University Insect Collections, Dept of Entomology, Cornell University, Ithaca NY (James Liebhner)

ESSIG: Essig Museum of Entomology, University of California-Berkeley, CA (Cheryl Barr)

JMC: J. M. Cicero Collection, Tucson, AZ

KSEM: The University of Kansas Natural History Museum, Snow Entomological Museum, Lawrence, KS (Robert Brooks)

KSH: Kathrin-Stanger Hall Collection, Austin, TX

OSU: Department of Entomology, Museum of Biological Diversity, Ohio State University, Columbus OH (Peter Kovarik)

SBMNH: Santa Barbara Museum of Natural History, Santa Barbara, CA (Michael Caterino)

Additional specimens were collected at localities throughout the southwestern United States during the summer months in 2001-2003. Adult males were collected with ultraviolet light traps and pitfall traps "baited" with light emitting diodes set up to mimic the females' advertising signal (Branham 2003). Specimens were killed in 95% ethanol and stored at -80° C.

Genitalic Dissection and Examination

When available, at 10 male specimens per locality were examined. In cases where fewer than 10 individuals were available, at least half of the available male specimens were examined. Terminal abdominal segments were removed with forceps and soaked in warm 10% potassium hydroxide until genitalia could be easily exerted. Male genitalia were rinsed in 95% ethanol and stored in glycerin for examination under a stereo dissecting scope at 50X power.

Morphological Characters and Character States

Three hundred and seventeen individual male specimens (66 *M. octarthrus*, 99 *M. angustus*, 6 *M. chiricahuae*, 10 *M. decarthrus*, 5 *M. fragilis*, 48 *M. pecosensis*, and 83 *M. dilatatus*) were examined. Sixteen characters traditionally used in species delimitation (Green 1959, Table 1, Figs. 1 & 2) were examined and coded in an individual by character matrix.

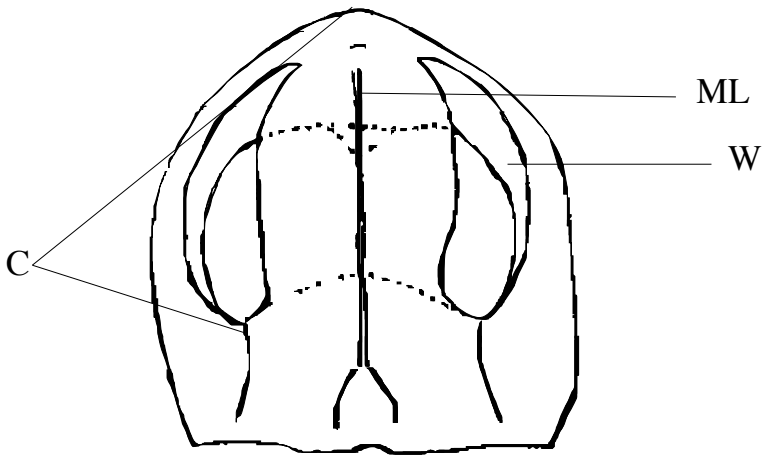

Fig 1. Pronotum of *M. fragilis*. C= convex area, ML=Median logitudinal line, W=windows (transparent spots)

Table 1. Morphological characters used for phylogenetic analysis

1. Number of antennomeres, including scape, pedicel and individual flagellomeres (0=less than 8, 1=8, 2=9, 3=10)

2. Eyes contiguous behind mouthparts, measured ventrally from behind the mouthparts to the back of the head (0=greater than half distance, 1=less than half distance)

3. Elytral length, measured from base to apex (0=greater than 3 times the length of pronotum, 1= less than 3 times the length of pronotum)

4. Elytral color (0=pale with dark tips, 1=uniform color)

5. Elytral shape (0=explanate, 1=parallel sided)

6. Pronotal size/ shape, measured laterally at widest point and from apex to base at widest point (0=wider than long, 1=as wide as long)

7. Pronotal base (0=truncate, 1=emarginate)

8. Medial longitudinal line of pronotum (0=impressed, 1=not impressed) (Fig. 1, ML)

9. Transparent spots on pronotum over eyes (0=present, 1=absent) (Fig. 1, W)

10. Circular convex area of pronotum, measured from apex of pronotum to the base of circular convex area (0= from apex to greater than 1/2 way to base, 1= from apex to less than 1/2 way to base) (Fig. 1, C)

11. Inner margins of lateral lobes of aedeagus, in dorsal view (0=converging toward apex, 1=diverging toward apex, 2= straight) (Figs. 2F, 2G, 2D)

12. Median lobe of aedeagus, in dorsal view (0=shorter than lateral lobe, 1=equal to length of lateral lobe, 2=longer than lateral lobe) (Figs. 2E, 2F, 2G)

13. Median lobe of aedeagus, in lateral view (0=visible above lateral lobes, 1=visible below lateral lobes, 2=visible between lateral lobes) (Figs. 2E, 2B, 2D)

14. Distal dorsal curvature of lateral lobe, in lateral view (0=no curvature, 1=concave, 2= convex) (Fig. 2G, 2C, 2B)

15. Lateral projections on medial lobe of aedeagus, in dorsal view (0=present, 1=absent) (Fig. 2 A, 2B)

16. Width of medial lobe, in dorsal view (0=uniform, 1=wider apically) (Fig 2D, 2A)

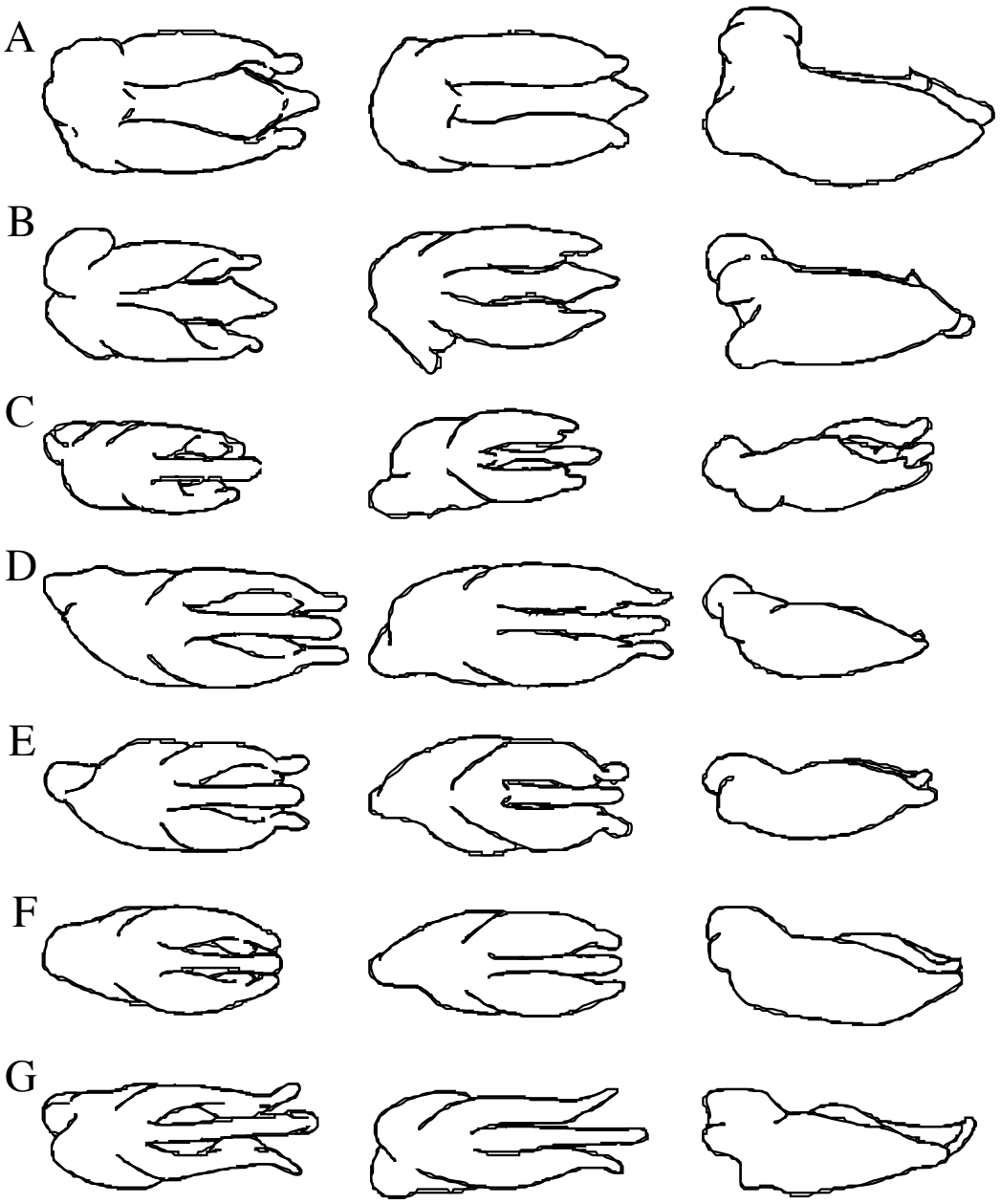

Fig 2. Genitalia of *Microphotus*. Dorsal, ventral and lateral view from left to right. A=*M. chiricahuae*, B=*M. pecosensis*, C=*M. octarthrus*, D=*M. dilatatus*, E=M. *deacrthrus*, F=*M. fragilis*, G=*M. angustus*

DNA Extraction, Amplification and Sequencing.

DNA was obtained from freshly collected specimens by pulling at least one thoracic leg and/ or thoracic muscle tissue. DNA from dried specimens was obtained from the entire thorax, which was ground with a conical stainless steel rod in a 1.5 ml microfuge tube. In both cases, DNA was extracted using Qiagen's DNeasy kit following the manufacturer's protocols. 2μL of extracted DNA from each specimen were prepared for polymerase chain reaction by addition of 35 μL of pure water, 5 μL of 5X $MgCl_2$-free Promega buffer, 4 μL of 25 mM Promega $MgCl_2$, 1 μL of 40 mM dNTPs, 0.2 μL of 100 U Promega *Taq* polymerase, and μL of 5 mM solution of each PCR primer, or through the addition of .19 μL of pure water and 2 μL of 5mM solution of each PCR primer to puRe Taq Ready-To-Go PCR Beads (Amersham Biosciences). Primers C1-J-2441 (alias Dick, CCAACAGGAATTAAATTTTAGAGATTAGC) and TL2-N-3014 (Pat) were used to amplify approximately 500 base pairs of the cytochrome c oxidase subunit I region of the mtDNA. Primers ATTCTGACTACCCAGATGTCTACTC (Mike) and TL2-N-3014 (Pat) were used to amplify approximately 200 base pairs of the same region(Simon et al 1994). PCR primers, AAGAGGTATGCACAGGTTCCAGG (Luc 1) and TAAGTGCTGTTGCTGTTTCGCG (Luc 2), were designed based on *Pyrocoelia rufa* cDNA luciferase sequence (GenBank accession number AF328553, Lee et al., 2001) and used to amplify approximately 750 base pairs of the luciferase gene. This region includes 2 introns of approximately 95- and 50-bp. Mitochondrial COI DNA sequences were amplified via PCR in a Peltier thermal cycler (PTC-200) using the following conditions: an initial denaturation at 95°C for 150 s, annealing at 45°C for 30

s, and extension at 72°C for 60 s for a total of 36 cycles, followed by 72°C extension for 5 min. Luciferase sequences were amplified via PCR in a Peltier thermal cycler (PTC-200) under the following conditions: 95°C for 150 s, 55°C for 30 s, and 72°C for 60 s for a total of 36 cycles, followed by 72°C for 5 min.

All PCR products were electrophoresed in a 1X Tris borate-EDTA buffer at 100 V for 30 min in 1.5% agarose gel stained with ethidium bromide (10 mg/ ml solution) and visualized with UV light. Unincorporated dNTPs and primers were removed with either a Qiagen PCR cleanup kit or an of EXO-SAP solution (USB product #70996). Five microliters of PCR product were added to 1 μL of EXO and 1 μL of SAP. The EXO-SAP cocktail was heated in a Peltier thermal cycler (PTC-200) at 37°C for 15 min followed by 80°C for 15 min. Cycle sequencing was performed with flourescently dyed terminator nucleotides (Big Dye kit, Applied Biosystems, Foster City CA) in a cocktail of 8 μL of PCR grade water, 2 μL of Big Dye, 3 μL of primers, and 2 μL of cleaned PCR product. Both strands of PCR product were sequenced. Cycle sequencing products were cleaned with 10% Sephadex solution and then visualized on an ABI 377 automated sequencer (Applied Biosystems).

DNA Sequence Analysis

The chromatographs of complementary DNA were edited into consensus sequences with Sequence Navigator. Alignment of individual sequences was straightforward as amino acids were conserved thus no nucleotide deletions or insertions were needed to maintain positional homology of nucleotides.

Phylogenetic Reconstruction

A data matrix for the morphological characters listed above was created using MacClade 4.0. The data matrix was analyzed in PAUP (Swofford 1998) under a maximum parsimony framework. A heuristic search of potential trees was performed with 35 replicates of random stepwise addition and branch swapping via subtree-pruning-regrafting. All other settings were default (all characters are of type 'unord', all characters have equal weight, multistate taxa interpreted as uncertainty, starting tree(s) obtained via stepwise addition, steepest descent option not in effect, branches collapsed (creating polytomies) if maximum branch length is zero, MulTrees' option in effect, topological constraints not enforced, trees are unrooted). Bootstrap values were obtained with 1000 replicates and default PAUP settings. The data matrix was also analyzed using the parsimony ratchet in NONA. 1 tree was held and 1 character sampled for 200 iterations. All other settings were default.

An additional optimal tree search for the combined COI and luciferase data sets was carried out in PAUP (Swofford 1998) under a maximum parsimony framework. A heuristic search of potential trees was performed with 1000 replicates of random stepwise addition, branch swapping via tree-bisection-reconnection, and default settings As described above. Molecular data were missing for *M. decarthrus*.. Only 192 bp of COI were included for *M. dilatatus*. Luciferase sequences were missing for *M. dilatatu*s, *M. octarthrus* 7 DR, and *M. octarthus* NC. Bootstrap values were obtained with 10000 random stepwise addition replicates via TBR branch swapping and default

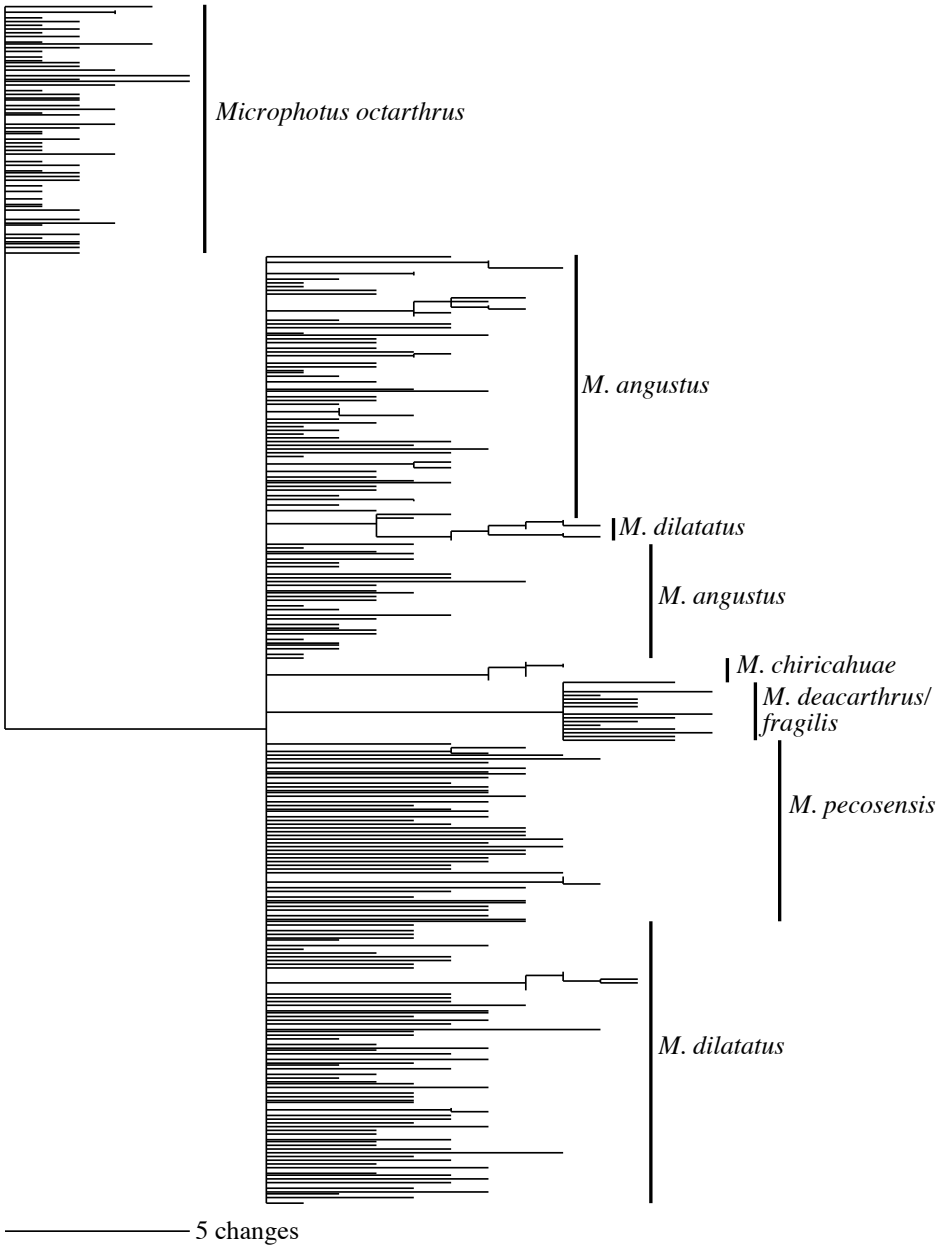

Fig.3 Phylogeny of *Microphotus* individuals based on morphological data. The tree shown is a strict consensus of 5000 trees (CI=0.445, RI=0.6626). Branches represent individuals.

settings as described above. Partitioned Bremer support was determined with TreeRot v.2 (Sorenson, 1999).

RESULTS

Species determination remains problematic because morphological characters are polymorphic. Within *M. dilatatus*, *M. angustus*, and *M. octarthrus*, the number of antennomeres varies between individuals. *M. dilatatus, M. angustus,* and *M. octarthrus* have eight to nine, nine to ten, and seven to eight antennomeres, respectively. In addition, some specimens within these species have asymmetrical antennae, in which one antenna is one flagellomere longer than the other. Elytral characters vary among individuals. Both dark elytral tips and uniform elytral color occur among populations of nearly all *Microphotus* species. In addition, parallel-sided and explanate elytral shapes occur within all *Microphotus* species. Continuity of the eyes behind the mouthparts, pronotal shape and size, the shape of the pronotal base, and impression of the medial longitudinal line of the pronotum all vary among individuals of nearly all species. Furthermore, a large range of variation of the genitalic characters was found.

Cladistic analysis of 16 morphological characters for 317 individuals yielded 5000 trees of length 279. A strict consensus of these trees yielded little resolution among the named species except for *M. octarthrus*.. The characters exhibited much homoplasy; consistency index was 0.0445. Monophyly of *M. octarthrus* is supported by a unique set of homoplastic characters. Absence of monophyly for the remaining species fails to reject the hypothesis that the characters reflect taxonomic limits (Fig. 3). Phylogenetic reconstruction using molecular data improved resolution among the species, which was a likely consequence of the increase of parsimony informative characters to 155. Simultaneous analysis of 470-bp of mtCOI gene, 755-bp of luciferase

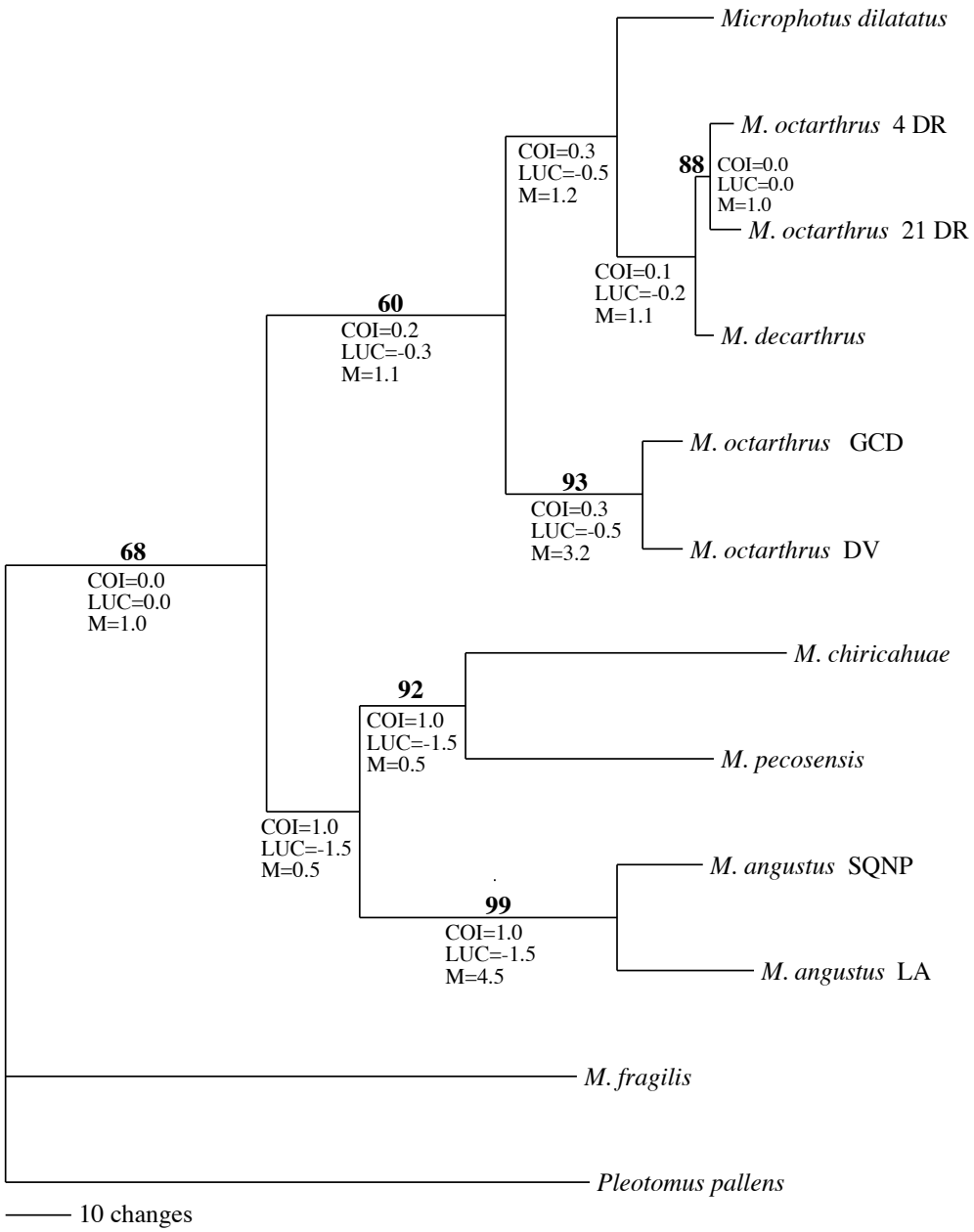

Fig.4 Phylogeny of *Microphotus* species based on combined morphological and molecular data. The tree shown is 1 of 2 most parsimonious trees for combinedmorphological and molecular data. CI=0.07861 , RI=0.6556. Bold numbers above branches indicate bootstrap values. Bootsyrap values less than 50 indicate clades unresolved in strict consensus. Numbers below branches indicate partitionedBremer support (COI= mt cytochrome oxidae I, LUC = luciferase, M = morphology). Numbers following taxa indicate individuals. Letters following taxa indicate populations (GCD=New Mexico, Catron Co., Gila Nat'l. For.; NC= Arizona, Graham Co., Coronado Nat'l. Forest., Noon Creek; DR=Texas, Val Verde Co., Devils River State NaturalArea; SQNP= California, Tulare Co., Sequoia Nat'l. Park, Potwisha; LA= California, Los Angeles Co., Los Angeles)

gene and 16 morphological characters yielded 2 trees of length 533 (CI= 0.07861, RI=0.6556) (Fig. 4). Nucleotides were mostly comprised of AT (mean = 0.682) and the

overall transition/transversion ratios for COI and luciferase are 2.16 and 1.0, respectively. Despite the observed nucleotide substitution biases, these data were not saturated as indicated by the linear relationship between JC and TN values (Fig. 5).

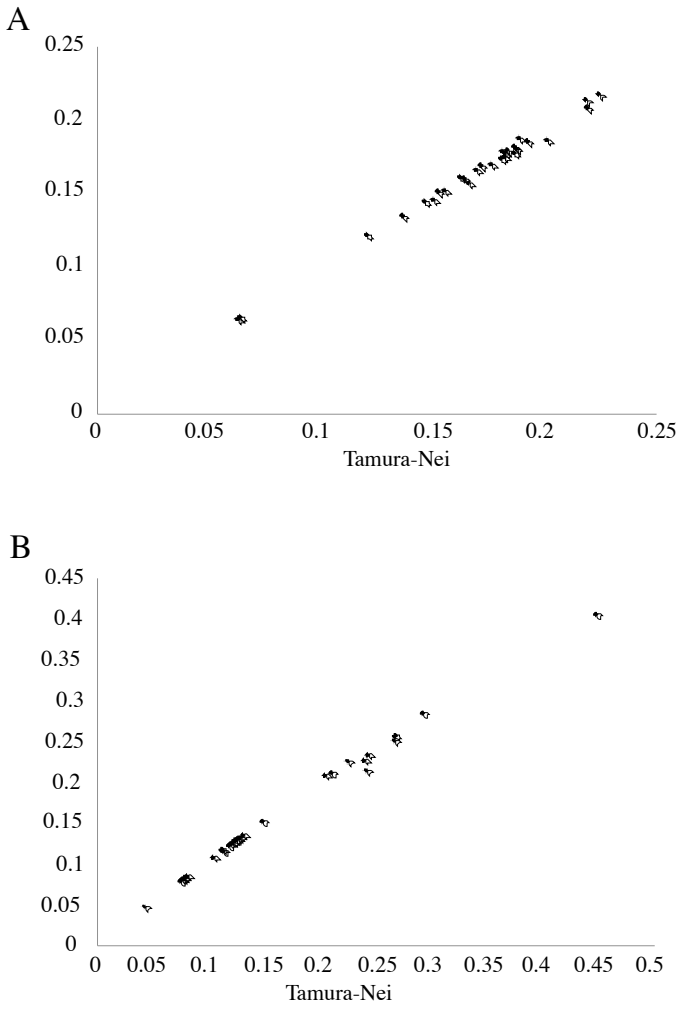

Fig.5 Tamura-Nei vs. Jukes-Cantor pairwise genetic distances. A=mtCOI, B=luciferase

In total 155 characters were phylogenetically informative; COI, luciferase and morphology exhibited 87, 52 and 16 respectively. Although bootstrap values > 60%

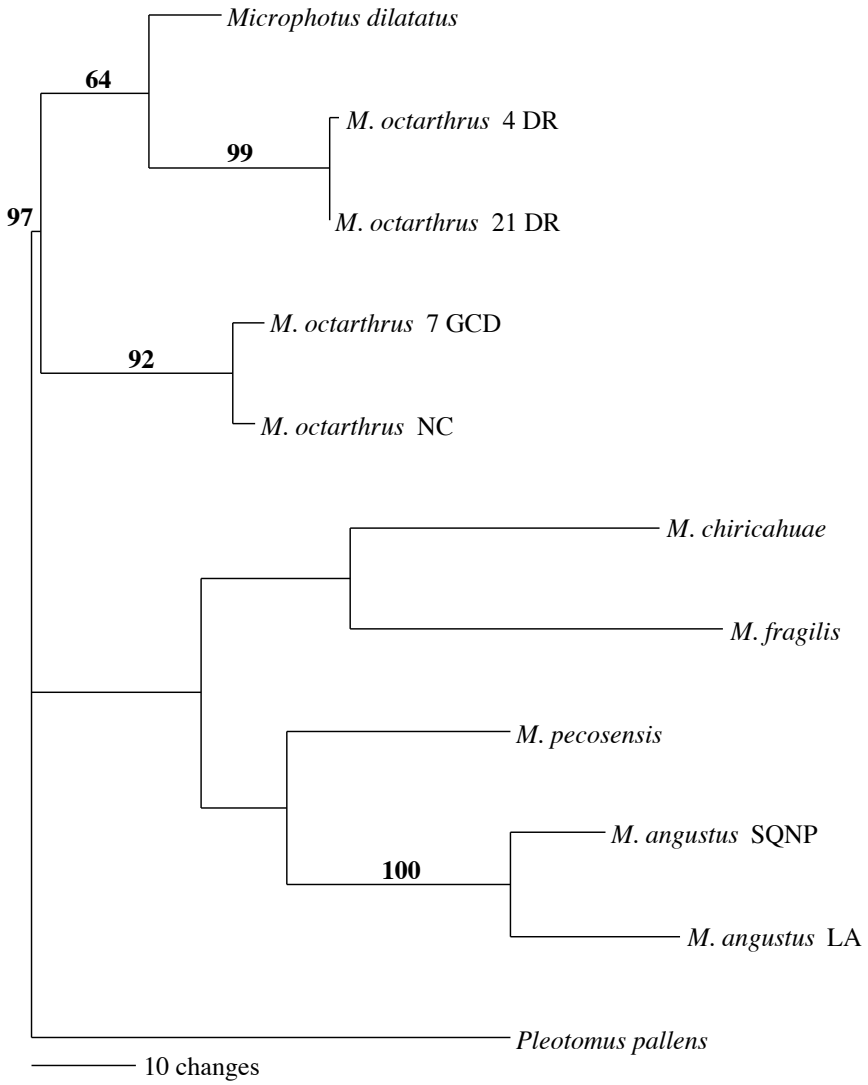

Fig. 6 Phylogeny of *Microphotus* species based on mtCOI data. The tree shown is 1 most parsimonious tree. CI=0.6611 , RI=0.6164. Bold numbers above branches indicatebootstrap values. Numbers following taxa indicate individuals. Letters following taxa indicate populations (GCD= New Mexico, Catron Co., Gila Nat'l. For.; NC= Arizona, Graham Co., Coronado Nat'l. Forest., Noon Creek; DR=Texas, Val Verde Co., Devils River State Natural Area; SQNP= California,Tulare

were found for the majority of clades, support differed for each data set. For example, COI and morphological data exhibited relatively higher amounts of partitioned Bremer support as compared to luciferase. It is surprising that luciferase conflicted with the

other data sets as observed by the negative Bremer support. This might have been a result of missing data from several of the OTUs however the effect of missing data on PBS is unexplored (Damgaard & Cognato 2003). Thus we analysized the COI and luciferase data sets separately and together using the same tree search criteria as above. Cladistic analysis of mtCOI yielded one most parsimonious tree of length 258 (CI=0.6611, RI=0.6164). Bootstrap values > 60% were recovered for less than half the clades; M. *chiricahuae, M. fragilis*, and *M. pecosensis* are poorly resolved (Fig 6). Cladistic analysis of luciferase yielded 1 most parsimonious tree of length 204 (CI=0.9020, RI=0.7436). Bootstrap values > 50 % were recovered for a majority of clades, and *M. pecosenis* and *M. chiricahuae* are better resolved (Fig 7). The simultaneous analysis of genes yielded 2 most parsimonious trees of length 470 (CI= 0.8170, RI=0.6627). Bootstrap values greater than 50% were observed for a majority of clades. Negative PBS values for basal clades indicate conflict in the data sets. Conflict is not observed in the peripheral clades, and luciferase gives the most support to these clades of the phylogeny (Fig 8).

DISCUSSION

The low CI associated with the morphological trees indicates a high amount of homoplasy among morphological characters. This indicates that the characters used by Green to define species limits with in *Microphotus* are traits rather than characters, and combinations of homplasies rather than synapomorphies that diagnose species. Based morphological characters and collection locality labels, there is little evidence that *M. decarthrus* and *M fragilis* are separate species. In addition, previous taxonomic

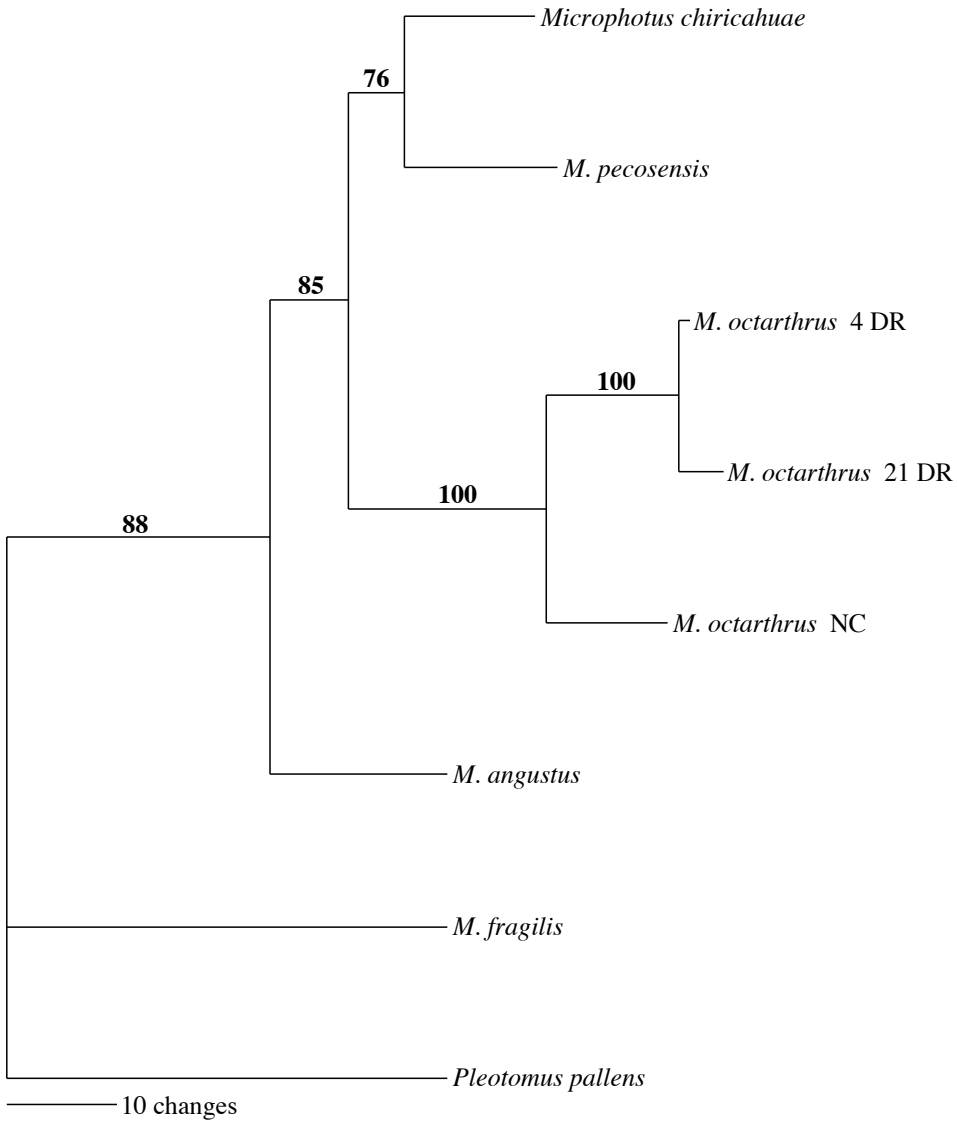

Fig. 7 Phylogeny of*Microphotus* species based on luciferase data.The single most parsimonius tree obtained is shown above. CI=0.9020 , RI=0.7436. Bold numbers above branches indicatebootstrap values. Numbers following taxa indicate individuals. Letters following taxa indicate populations (NC= Arizona, GrahamCo.Coronado Nat'l. Forest., Noon Creek;

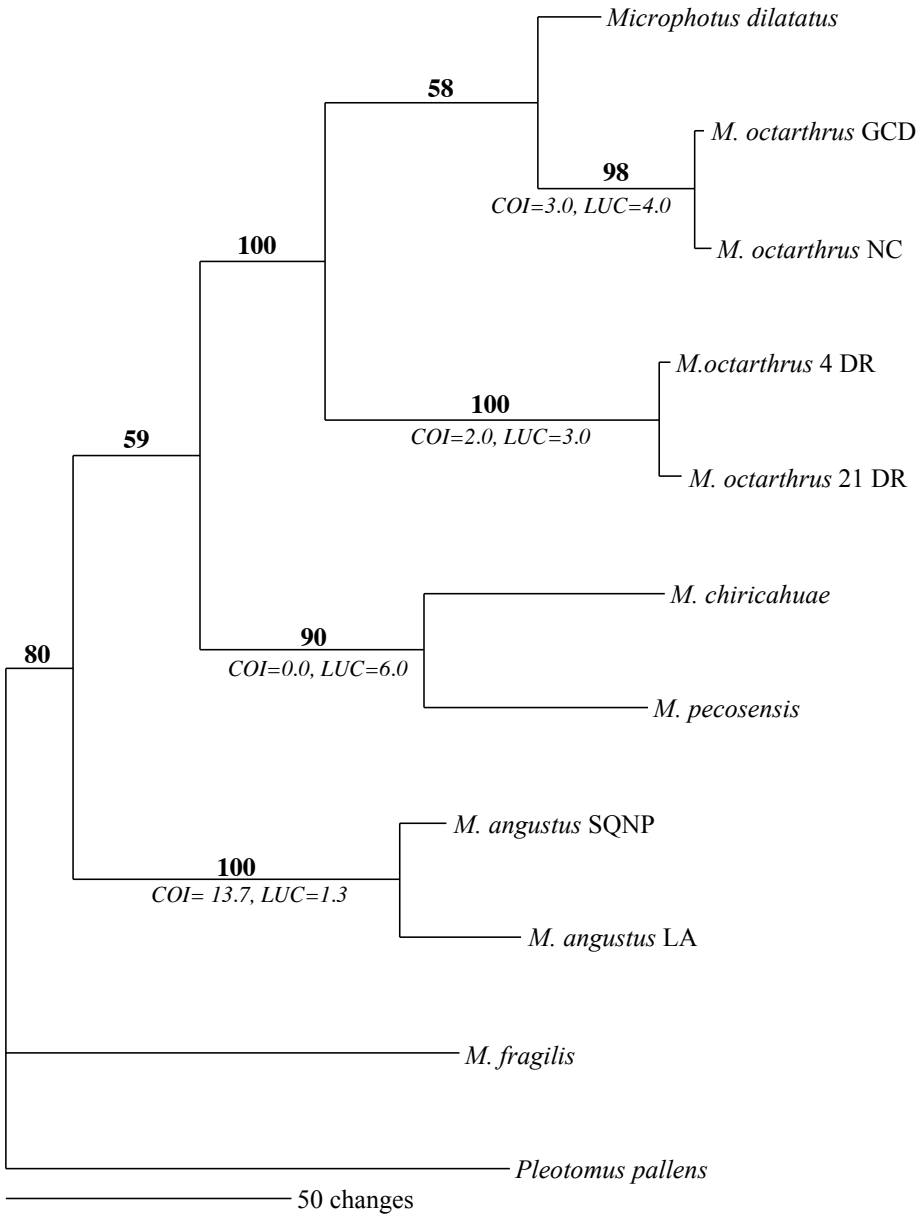

Fig. 8 Phylogeny of *Microphotus* species based on combined molecular data. The treee shown is 1 of 2 most parsimonious trees for combined mtCOI and luciferase data. CI= 0.8170, RI=0.6627. Bold numbers above branches indicate bootstrap values. Bootstrap values less than 50 indicate clades unresolved in strict consensus. Italicized numbers below branches indicate partitionedBremer support (COI= mt cytochrome oxidae I, LUC = luciferase). Numbers following taxa indicate individuals. Letters followingtaxa indicate populations (GCD=New Mexico, Catron Co., Gila Nat'l. For.; NC= Arizona, Graham Co., Coronado Nat'l. Forest., Noon Creek; DR=Texas, Val Verde Co., Devils River State Natural Area; SQNP= California,Tulare Co., Sequoia Nat'l. Park, Potwisha; LA= California, Los Angeles Co., Los Angeles)

observations suggest the synonymy of these species (Green 1959). Molecular data may diagnose these species however the lack of fresh specimens precluded further analysis. Nevertheless, given the taxonomic confusion and problematic diagnosis of these two species, *M. fragilis* is here synonomized with *M. decarthrus*. (syn. nov.)

Until a more thorough study including behavior, more genes, populations, and individuals, is undertaken, the remaining *Microphotus* species should be recognized. Although morphological characters appear to be mostly homoplastic, unique combinations these characters still allow for the diagnosis of currently defined species. Synonomizing or describing addition species would only further confound species limits.

Reproductive barriers are expected to exist among *Microphotus* species given that many species occur in sympatry. It has been suggested that mating behavior including female advertising posture, male approach, and coupling time are potential reproductive barriers and may useful in species delimitation (Cicero, 1981). However, sex ratios among *Microphotus* species appear to be highly disproportionate (Cicero, personal communication), and females are encountered less frequently than males. This has limited the study of mating behavior only to one population in each of three (Cicero, 1981). Other species, such as *M. angustus* and *M. chiricahuae* are relatively restricted in distribution. Although it is expected that species these species would be less isolated by distance, the patchwork of suitable habitat within California may effectively isolate populations of *M. angustus*. Morphological differences in genitalia are observed among *M. angustus* individuals of different populations. These differences may indicate cryptic

species but integration of these characters is observed among *M. angustus* and *M. dilatatus* individuals.

Molecular data has resolved species limits for many insect species resulting in taxonomic revision (Morgan & Vogler 2000). Our data suggest that both COI and luciferase provide many characters that will help resolve a phylogeny of *Microphotus* species (Fig 5). As advocated for morphological taxonomic characters (Vrana & Wheeler 1992) and as demonstrated in this study, species limits and taxonomy of *Microphotus* may best be determined through the phylogenetic analysis of individuals. Molecular data would need to be generated for hundreds of individuals sampled through species distributions. However, this endeavor is limited by the infrequency of live specimens.

CHAPTER III

POPULATION GENETICS PATTERNS FOR *M. octarthrus* Fall BASED ON mtCOI DNA SEQUENCE

Allopatric speciation occurs when geographic isolation results in reproductive isolation (Mayr 1969). Gene flow is prevented between populations, resulting in genetic divergence. In this scenerio, increased time in isolation presents more opportunity for evolution of pre- and/or post mating barriers. Current disjunct populations may have only a 10,000 year history since the warming and deglaciation of North America and may have experienced 100,000 years of geography continuity (Webb & Bartlein 1992). . Thus the absence of allopatric barriers would have maintained gene flow and species cohesiveness among current disjunct populations. Whether or not 10,000 years is enough time for speciation to occur is debatable (Mutun & Borst 2004, Vandyke at al 2004, Tregenzat et al. 2002).

A desert firefly, *Microphotus octarthrus* Fall, exemplifies this scenario. This species is widespread throughout the southwestern United States and occurs in Pinyon-Juniper and Juniper-Oak habitats between 500- 2000 m in Arizona, New Mexico, west Texas, and Utah. Males of this species are fully winged. The apterous larva-like females emit glows from photic organs on the underside of the abdomen in sexual communication. These beetles appear to have a highly disproportionate sex ratio, and males are encountered more frequently than females (Cicero, personal communication). Genetic variation within *Microphotus* may be isolated, given the limited dispersal of

larva-like females and discontinuous pinyon-juniper habitat. Segregation of genetic variation in disjunct populations may suggest cryptic species (Cognato 2000, Vandyke et al 2004). Cytochrome oxidase I has been used extensively for beetles especially when examining closely related species and populations (Caterino et al 2000) and represents a neutral marker. Nucleotide data for mitochondrial cytochrome oxidase I gene is used to examined genetic variation among disjunct populations of *Microphotus octarthrus*.

MATERIALS AND METHODS

DNA was obtained from freshly collected specimens by pulling at least one thoracic leg and/ or thoracic muscle tissue. DNA from dried specimens was obtained from the entire thorax which was ground with a conical stainless steel rod in a 1.5 ml microfuge tube. In both cases, DNA was extracted using Qiagen's DNeasy kit following the manufacturer's protocols. 2μL of extracted DNA from each specimen were prepared for polymerase chain reaction by addition of 35 μL of pure water, 5 μL of 5X $MgCl_2$-free Promega buffer, 4 μL of 25 mM Promega $MgCl_2$, 1 μL of 40 mM dNTPs, 0.2 μL of 100 U Promega *Taq* polymerase, and μL of 5 mM solution of each PCR primer. PCR primers C1-2183 (alias Jerry, CAACATTTATTTGATTTTTTGG) and TL2-N-3014 (alias Pat, TCCATTGCACTAATCTGCCATATTA) were used to amplify approximately 700 base pairs of the cytochrome c oxidase subunit I region of the mtDNA. Primers C1-J-2441 (alias Dick, CCAACAGGAATTAAATTTTAGAGATTAGC) and TL2-N-3014 (Pat) were used to amplify approximately 500 base pairs of the same region. Primers ATTCTGACTACCCAGATGTCTACTC (Mike) and TL2-N-3014 (Pat) were used to

amplify approximately 200 base pairs of the COI region of the mtDNA (Simon et al 1994). PCR was carried out in a Peltier thermal cycler (PTC-200) under the following conditions: an initial 95°C for 150 s, 45°C for 30 s, and 72°C for 60 s for a total of 36 cycles, followed by 72°C for 5 min.

All PCR products were electrophoresed in a 1X Tris borate-EDTA buffer at 100 V for 30 min in 1.5% agarose gels stained with ethidium bromide (10 mg/ ml solution) and visualized with UV light. PCR products were then cleaned of unincorporated dNTPs and primers through use of a Qiagen PCR cleanup kit following the manufacturer's instruction or through the use of EXO-SAP solution (USB product #70996). 5 μL of PCR product were added to 1 μL of EXO and 1 μL of SAP. The EXO-SAP cocktail was heated in a Peltier thermal cycler (PTC-200) at 37°C for 15 min followed by 80°C for 15 min. Cycle sequencing followed and was performed with flourescently dyed terminator nucleotides (Big Dye kit, Applied Biosystems, Foster City CA) in a cocktail of 8 μL of PCR grade water, 2 μL of Big Dye, 3 μL of primers, and 2 μL of cleaned PCR product. Both strands of PCR product were sequenced. Cycle sequencing products were cleaned with 10% Sephadex solution and then visualized on an ABI 377 automated sequencer (Applied Biosystems).

DNA Sequence Analysis

The chromatographs of complementary DNA were edited into consensus sequences with Sequence Navigator. Alignment of individual sequences was straightforward as amino acids were conserved thus no nucleotide deletions or insertions were needed to maintain positional homology of nucleotides.

Phylogenetic Reconstruction

An optimal tree search for COI was carried out in PAUP (Swofford 1998) under a maximum parsimony framework for 28 *Microphotus octarthru*s individuals, four additional *Microphotus* species (*M. pecosensis, M. angustus, M. chiricahuae, and M. fragilis*.), and outgroup species (*Photinus pyralis*, *Pleotomus pallens*.). A heuristic search of potential trees was performed with 50 replicates of random stepwise addition and branch swapping via tree-bisection-reconnection. All other settings were default (all characters are of type 'unord', all characters have equal weight, multistate taxa interpreted as uncertainty, starting tree(s) obtained via stepwise addition, steepest descent option not in effect, branches collapsed (creating polytomies) if maximum branch length is zero, MulTrees' option in effect, topological constraints not enforced, trees are unrooted). Bootstrap values were determined with 10000 replicates via fast-heursitc search and default PAUP settings as described above.

Geographical and Genetic Distance Comparison

Pairwise genetic and geographical distances were calculated for *M. octarthrus* mtCOI DNA haplotypes. Genetic distances were calculated under a Jukes-Cantor nucleotide evolution model in PAUP (Jukes & Cantor 1969). The geographical distances (in kilometers) between collection localities were determined with an online distance calculator (http://www.indo.com/cgi-bin/dist). The R Package computer program (Casgrain & Lengendre 2001) was used to associate genetic and geographic distances with Mantel's approximate t-test.

RESULTS

Cladistic analysis of 771 bp of the mtCOI gene yielded 15500 trees of length 627 (CI=0.7024, RI=0.7536). The strict consensus tree was mostly resolved and exhibited relatively high bootstrap values. A total of 26 mtDNA COI haplotypes was found. Haplotypes were unique for Texas, Utah, northern New Mexico and southeastern Arizona/ southwestern New Mexico. Among Texas haplotypes, unique haplotypes existed for Big Bend National Park, Devils River State Natural Area and Davis Mountains State Park. A significant isolation by geographic distance (Mantel t = 5.719670, P = 0.000000) existed among these haplotypes. Pairwise nucleotide Jukes-Cantor distances among *M. octarthrus* individuals ranged between 0 and 12% (mean=6.4%). Percent sequence divergence as measured in terms of branch length among designated clades of *M. ocarthrus* (Fig. 9, clades A &B) ranged between 0.97 and 5.2% (mean=2.6%). Percent sequence divergence as measured in terms of branch length among *Microphotus* species (Fig 9, clade C), excluding *M. octarthrus* ranged between 3.2 and 4.5% (mean=4%).

DISCUSSION

The reconstructed phylogeny suggests greater species diversity among populations of *M. octarthrus*, and supports the hypothesis of genetically isolated populations (Fig 9). Texas, northern New Mexico, and southeastern Arizona/ southwestern New Mexico/Utah populations are monophyletic. The mean branch lengths as measured from the individual to the root of the Texas and Northern New Mexico groups are 4.35% and 5.0%, respectively (Fig 9 A&C). These values are similar to the

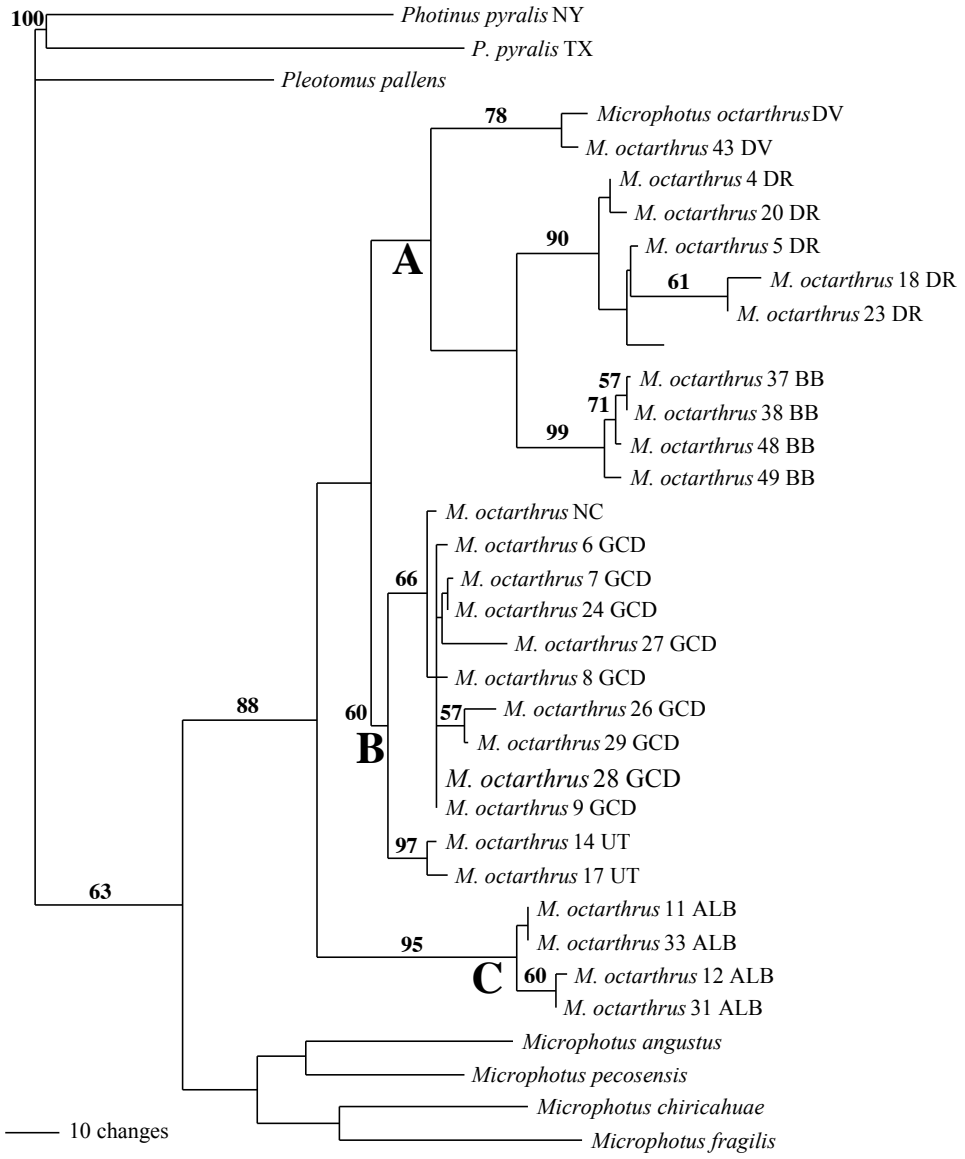

Fig. 9 Phylogeny of *M. octarthrus* populations based on mtCOI data. The treee shown is 1 of 15500 most parsimonious trees. CI=0.7024, RI=0.7536. Bootstrap values are given in bold abovebranches. Clades with bootstrap values lower than 50 are unresolved in strict consensus. Numbers following taxa indicate individuals. Letters following taxa indicate populations (NY=New York, Suffolk Co, Smithtown; TX=Texas, Fort Bend Co., Brazos Bend St. Park; DV=Texas, Jeff Davis Co., Davis Mts. St. Park; DR=Texas, Val Verde Co., Devil's River State Natural Area; BB= Texas, Presidio Co., Big Bend Nat'l. Park; NC=Arizona, GrahamCo., Coranado Nat'l Forest, Noon Creek; GCD=New Mexico, Catron Co., Gila Nat'l. For.;UT=Utah,Washington Co., nr. Baker Dam Resevoir ; ALB=New Mexico, Bernalillo Co., nr. Albuquerque)

differences observed in among the other *Microphotus* species. Given an average mitochondrial sequence divergence of 2.3% per million years (Brower 1994), isolation

of these groups of populations occurred approximately 1.89, 2.17 and 1.7 million years ago, which coincides with the beginning of the Pleistocene. Thus, segregation of these populations began long before the start of the Holocene. The preferred habitat was likely discontinuous throughout the Pleistocene, although the pinyon- juniper/oak-juniper habitats likely occupied lower altitudes and latitudes (Wells 1987).

Whether or not these populations represent cryptic species remains undetermined. Few morphological differences are observed between some of the populations examined. For example, members of the Devil's River clade (DR) generally have 7 or 8 antennomeres. However, as discussed in Chapter II, current morphological characters used in species delimitation exhibit high homoplasy. Behavioral differences such as female advertising posture, male approach, and coupling time would further support the isolation of these population via pre-mating barriers and recognition of new species. A more thorough sampling of populations, throughout the range *M. octarthrus* and examination of more genes are required to further elucidate the species limits within *M. octarthrus*. However, these data suggest that there is a diversity of "old" lineages of *M. octarthrus* and it is likely that a similar amount of diversity exists among the other five recognized *Microphotus* species given the similarity in behavior and ecology.

CHAPTER IV

CONCLUSION

Morphological characters, mtCOI DNA and luciferase DNA were examined for phylogenetic utility in defining *Microphotus* species limits. Analysis of 16 morphological characters for 317 individuals resulted in 5000 phylogenies with poor resolution among the named species, indicating unique combinations of homoplastic characters define *Microphotus* species. Due to the lack of variation seen in morphological characters and the proximity of collection localities to one another, *M. decarthrus* and *M. fragilis* are synonymized. All other Microphotus species should be recognized. Mitochondrial cytochrome oxidase I and luciferase DNA were examined for phylogenetic signal. Although some conflict exists between the data sets, both contribute to the phylogeny.

Mitochondrial COI DNA was also examined at the population level for *M. octarthrus*. Analysis revealed 26 haplotypes among 28 individuals. Unique clades exist for Texas, Utah, northern New Mexico, and southeastern/ southwestern New Mexico populations. Mantel's t-test approximation indicates these clades are geographically and genetically isolated. Some morphological difference is observed between populations, however, morphology appears to be too variable for species delimitation. Mean branch length for these clades indicate segregation of these populations began prior to the start of the Holocene.

The phylogeny obtained for *M. octarthrus* populations based on mtCOI hints at higher species diversity within this species. Given the similarities in behavior and

ecology, it is likely that a similar amount of diversity exists for the other five *Microphotus* species. However, more data, including a more thorough sampling through the range of genus, more genes, and behavior are needed to further elucidate the species limits with *Microphotus*.

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