SHORT COMMUNICATION

Molecular Phylogeny of *Chrysomya albiceps* and *C. rufifacies* (Diptera: Calliphoridae)

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ABSTRACT Mitochondrial DNA was used to infer the phylogeny and genetic divergences of *Chrysomya albiceps* (Wiedemann) and *C. rufifacies* (Maquart) specimens from widely separated localities in the Old and New World. Analyses based on a 2.3-kb region including the genes for cytochrome oxidase subunits I and II indicated that the 2 species were separate monophyletic lineages that have been separated for >1 million years. Analysis of DNA, in the form of either sequence or restriction fragment-length polymorphism (RFLP) data, will permit the identification of problematic specimens.

KEY WORDS *Chrysomya albiceps, Chrysomya rufifacies,* systematics, mitochondrial DNA, cytochrome oxidase, forensic entomology

THE RECENT INVASION of the Americas by several *Chrysomya* species (Baumgartner and Greenberg 1984) includes 2 forms that represent an old taxonomic problem. Until recently, *C. albiceps* was known to occur from northwestern India to southern Africa, and *C. rufifacies* was known from India to the islands of the Pacific. Although both have been reported from what is now southern Pakistan (Senior White et al. 1940), and very recently from Iran (Parchami-Araghi 1995), the amount of contact between the Old World populations has not been described. Introduced at opposite ends of Latin America (*C. albiceps* in Brazil and *C. rufifacies* in Costa Rica), reports now indicate that their ranges overlap in the New World (see references in Tantawi and Greenberg 1993).

These flies have been regarded alternately as the same species (Zumpt 1965, Ullerich 1963, Kurahashi 1989) or different species (Holdaway 1933, Tantawi and Greenberg 1993). Morphological characters separating the 2 have been described, *C. rufifacies* is distinguished from *C. albiceps* by the presence of a proepisternal (=stigmatal) bristle in the adult and spines on the shafts of larval tubercles (Holdaway 1933, Erzinclioglu 1987). However, these and some other differences are minor and, in a small percentage of individuals, variable (Zumpt 1965, Tantawi and Greenberg 1993). Furthermore, they can interbreed to produce fertile hybrids in the laboratory (Ullerich 1963), and the natural histories of the 2 forms appear to be identical (Zumpt 1965).

Although the ability to interbreed in nature is a standard criterion for including 2 organisms in the same species (Mayr 1942), hybridization that is possible in captivity does not necessarily occur in the wild. Two pairs of calliphorid species that have produced fertile hybrids are *Lucilia cuprina* Wiedemann and *L. sericata* Meigen (Mackerras 1933), and *Chrysomya megacephala* (F.) and *C. pacifica* Kurahashi (H. Kurahashi, personal communication). Despite this behavior in the laboratory, there is no evidence that either pair of species interbreed when they overlap in the field. A world-wide survey of *Lucilia* specimens found that even sympatric *L. cuprina* and *L. sericata* could always be distinguished based on molecular-genetic markers (Stevens and Wall 1997). J.D.W. observed hundreds of *C. megacephala* and *C. pacifica* on the same baits placed at the intersection of their respective habitats in New Britain, Papua New Guinea, but found none of the intermediate forms produced when they were crossed in the laboratory.

Resolving the status of *C. albiceps* and *C. rufifacies* is of interest to more than just calliphorid taxonomists. The *Chrysomya* species that have invaded the Americas are of applied importance and have had a dramatic negative effect on the native fly fauna. As a result, they have stimulated a wide range of medical, ecological, and forensic studies (e.g., Baumgartner and Greenberg 1984, Mariluis and Schnack 1989, Wells and Greenberg 1994, Byrd and Butler 1997, De Souza and Linhares 1997, Godoy et al. 1997, [and many others]). Unambiguous species diagnoses, particularly of immature stages, are of primary importance to forensic entomologists and will be invaluable for any future studies of the ecological and genetic interaction between *C. albiceps* and *C. rufifacies* in Latin America.

Given the taxonomic ambiguity that exists despite considerable morphological and ecological investigation, a new class of characters is needed to resolve this problem. Animal mitochondrial DNA (mtDNA) is no-

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Table 1. Primers used in th

Location[a]	
1. TY-J-1460	T
2. C1-N-1687	C
3. C1-J-1709	A
4. C1-J-1751a	G
5. C1-J-1751b	G
6. C1-N-1840	A
7. C1-J-2183	C
8. C1-N-2191	C
9. C1-N-2293a	A
10. C1-N-2293b	A
11. C1-N-2329	A
12. C1-J-2495	C
13. C1-N-2659	G
14. C1-J-2792a	A
15. C1-J-2792b	A
16. TL2-N-3013	T
17. C2-J-3138	A
18. C2-N-3389	T
19. C2-J-3408	C
20. TK-N-3775	C
C. albiceps	
Egypt	
S. Africa1	
S. Africa2	
Brazil	
C. rufifacies	
Texas	
Florida	
Australia	
Fr. Polynesia	
Indonesia	
Vietnam	

[a] Nomenclature of Simon et
[b] Purchased from Nucleic A
[c] Parentheses enclose pairs u

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We used mtDNA to
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Analyses were based c
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Materia

Adult *C. albiceps* we
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emfontein, Orange Fre
rufifacies were from M
originating in Kerrville
Australia; Moorea, Fre
Masohi, Ceram, Indor
Phu Province, Vietnam
rufifacies possessed pr

Table 1. Primers used in this study and combinations used for each specimen

Location[a]	Primers Sequence	Reference
1. TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC	Sperling et al. (1994)
2. C1-N-1687	CAATTTCCAAATCCTCCAATTAT	New
3. C1-J-1709	ATAATTGGAGGATTTGGAAATTG	New
4. C1-J-1751a	GGATCACCTGATATAGCATTCCC	Bogdanowicz et al. (1993)
5. C1-J-1751b	GGATCCCCTGATATAGCT/CTTTCC	New
6. C1-N-1840	AGGAGGATAAACAGTTCAC/TCC	Sperling et al. (1995)
7. C1-J-2183	CAACATTTATTTTGATTTTTTGG	Simon et al. (1994)
8. C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC	Bogdanowicz et al. (1993)
9. C1-N-2293a	AGTAAACCAATTGCTAGTATAGC	New
10. C1-N-2293b	ATGGCATAAATTATTCCTAAAGC	New
11. C1-N-2329	ACTGTAAATATATGATGAGCTCA	Commercial product[b]
12. C1-J-2495	CAGCTACTTTATGAGCTTTAGG	Sperling et al. (1994)
13. C1-N-2659	GCTAATCCAGTGAATAATGG	Sperling and Hickey (1994)
14. C1-J-2792a	ATACCTCGACGTTATTCAGA	Bogdanowicz et al. (1993)
15. C1-J-2792b	ATACCTCGGCGATACTCTGA	New
16. TL2-N-3013	TCCATTACATATAATCTGCCATATTAG	New
17. C2-J-3138	AGAGCCTCTCCTTTAATAGAACA	Simon et al. (1994)
18. C2-N-3389	TCATAAGTTCA[R]TATCATTG	Bogdanowicz et al. (1993)
19. C2-J-3408	CAATGATAT/CTGAAGT/ATATGA	New
20. TK-N-3775	GAGACCATTACTTGCTTTCAGTCATCT	Bogdanowicz et al. (1993)

	Combinations[c]
C. albiceps	
Egypt	(1,2) (1,6) (4,8) (7,13) (12,16) (14,18) (17,20) (19,20)
S. Africa1	(1,2) (1,6) (4,10) (7,13) (12,16) (14,18) (17,20) (19,20)
S. Africa2	(1,2) (1,6) (4,9) (7,13) (12,16) (14,18) (17,20) (19,20)
Brazil	(1,2) (1,6) (4,8) (7,13) (12,16) (14,18) (17,20) (19,20)
C. rufifacies	
Texas	(1,2) (1,6) (3,8) (3,11) (7,13) (12,16) (15,18) (17,20) (19,20)
Florida	(1,2) (1,6) (5,8) (7,13) (12,16) (14,18) (17,20) (19,20)
Australia	(1,2) (1,6) (5,8) (7,13) (12,16) (14,18) (17,20) (19,20)
Fr. Polynesia	(1,2) (1,6) (4,8) (7,13) (12,16) (14,18) (17,20) (19,20)
Indonesia	(1,2) (1,6) (4,8) (4,11) (7,13) (12,16) (14,18) (17,20) (19,20)
Vietnam	(1,2) (1,6) (4,8) (7,13) (12,16) (14,18) (17,20) (19,20)

[a] Nomenclature of Simon et al. (1994).
[b] Purchased from Nucleic Acid Service Unit, University of British Columbia.
[c] Parentheses enclose pairs used for individual PCR reactions.

table for the relative ease with which one can obtain sequence data and determine homologies, and for having regions that evolve quickly compared with nuclear DNA (Harrison 1989). For these reasons it is particularly useful for understanding phylogenetic relationships at or below the species level (Sperling and Hickey 1994), and for designing molecular-diagnostic tests for identifying specimens (Sperling et al. 1994).

We used mtDNA to infer the molecular-phylogenetic relationships of *C. albiceps* and *C. rufifacies* from widely separated localities in the Old and New World. Analyses were based on a 2.3-kb region coding for cytochrome oxidase subunits I and II as well as tRNA-leucine.

Materials and Methods

Adult *C. albiceps* were from Moharrem Bey, Alexandria, Egypt; Campinas, Sao Paulo, Brazil; and Bloemfontein, Orange Free State, South Africa. Adult *C. rufifacies* were from Miami, FL; a laboratory colony originating in Kerrville, TX; Adelaide, South Australia, Australia; Moorea, French Polynesia; Maluku Tengah Masohi, Ceram, Indonesia; and Mt. Tam Dao, Vinh Phu Province, Vietnam. All specimens referred to as *C. rufifacies* possessed proepisternal setae, and all *C. al-*

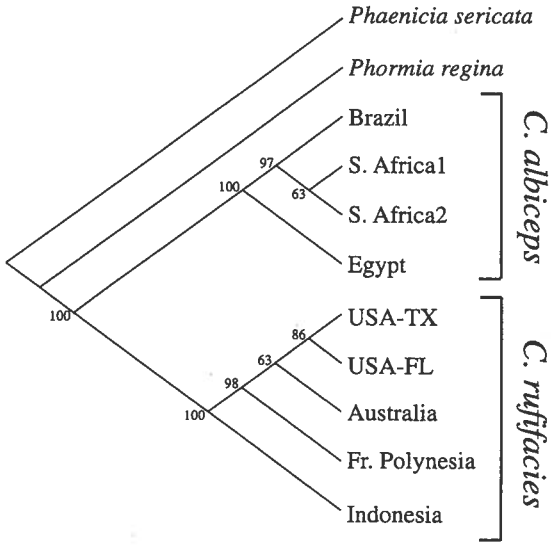

Fig. 1. Single most parsimonious phylogeny of *Chrysomya* specimens based on a 2.3-kb sequence of mitochondrial DNA. Numbers indicate bootstrap support for individual branches. The outgroups *Phaenicia sericata* and *Phormia regina* are from Sperling et al. (1994).

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tacaatttatcgcctaaacttcagcc--------ATTTAATCGCGACAATGGTTATTTTCTACTAATCATAAAGATATTGGTACTTTATATTTCATTTTCG 1534
GAGCTTGATCTGGAATAGTAGGAACTTCTTTAAGAATTCTAATTCGAGCTGAATTAGGACATCCTGGAGCACTAATTGGAGATGACCAAATTTATAATGT 1634
AATTGTAACAGCTCATGCCTTTATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAATTGACTAGTTCCTTTAATATTAGGAGCC 1734
CCAGATATAGCTTTCCCACGAATAAATAATATAAGTTTCTGACTTTTACCTCCTGCATTAACTTTACTATTAGTAAGTAGTATAGTAGAAAATGGAGCTG 1834
GAACAGGATGAACTGTTTATCCACCTTTATCATCTAATATTGCTCATGGTGGAGCATCAGTTGATTTAGCTATTTTTTCTTTACACTTAGCTGGAATTTC 1934
ATCAATTTTAGGAGCTGTAAATTTTATTACAACTGTTATTAATATACGATCTACAGGAATCACATTTGATCGAATACCTTTATTCGTATGATCTGTAGTT 2034
ATTACTGCTCTTCTTTTATTATTATCATTACCAGTATTAGCCGGTGCAATTACTATATTATTAACTGATCGAAATTTAAATACTTCATTCTTTGATCCAG 2134
CAGGAGGAGGAGATCCTATTTTATATCAACATTTATTTTGATTCTTTGGACATCCAGAAGTTTATATTTTAATTTTACCTGGATTCGGAATAATTTCTCA 2234
TATTATTAGTCAAGAATCAGGAAAAAAGGAAACATTTGGATCTTTAGGAATAATTTATGCAATATTAGCTATTGGTCTATTAGGATTTATTGTATGAGCT 2334
CATCATATATTTACTGTAGGAATGGATGTAGATACTCGAGCATATTTTACTTCAGCTACAATAATTATTGCTGTACCAACTGGAATTAAAATTTTTAGTT 2434
GATTAGCAACTCTTTATGGAACACAATTAAATTACTCCCCAGCTACCTTATGAGCTTTAGGATTTGTATTTTTATTTACTGTAGGAGGATTAACTGGAGT 2534
TGTTTTAGCTAATTCATCTATTGATATTATTTTACATGATACATATTATGTAGTAGCTCACTTCCATTATGTTCTTTCAATAGGAGCTGTATTCGCTATT 2634
ATAGCAGGATTCGTTCATTGATTCCCATTATTTACTGGATTAACTCTAAATAATAAAATACTAAAAAGTCAATTTGCTATTATATTTATTGGAGTAAATT 2734
TAACATTCTTTCCTCAACATTTTTTAGGATTAGCTGGTATACCTCGACGATACTCAGATTACCCAGATGCTTATACAGCATGAAATGTTATCTCAACAAT 2834
TGGGTCAACAATTTCATTATTAGGAATTTTATTTTTCTTTTTCATTATTTGAGAAAGTTTAGTATCTCAACGACAAGTTTTATTTCCTATTCAATTAAAT 2934
TCATCAATTGAATGACTTCAAAATACTCCTCCAGCTGAACATAGTTATAGTGAATTACCTTTATTAACTAATT----TCTAATATGGCAGATTAGTGCAA 3034
TGGATTTAAGCTCCATATATAAAGTATTTTACTTTTATTAGAA---TACAAATGTCAACATGAGCAAATTTAGGTTTACAAGATAGTTCTTCACCATTAA 3134
TAGAACAATTAATCTTTTTCCATGACCACGCACTTTTAATTTTAGTAATAATTACTGTACTAGTAGGTTATCTAATATTTATATTATTTTTTAATAAATA 3234
TGTAAATCGATATTTACTTCACGGACAAACCATTGAAATTATTTGAACAATTTTACCAGCAATTATTTTATTATTTATTGCTTTTCCTTCTTTACGATTA 3334
TTATATTTATTAGATGAAATTAATGAACCTTCTATTACTTTAAAGGCAATTGGACATCAATGATATTGAAGTTATGAATATTCAGATTTTGCTAATATTG 3434
AATTTGATTCATACATAATTCCTACAAACGAATTATCAATTGATAGATTCCGTTTATTAGATGTTGATAATCGAGTAGTTTTACCTATAAATTCACAAAT 3534
TCGAATTTTAGTGACAGCAGCTGACGTAATTCATTCATGAACTATCCCAGCTTTAGGAGTTAAGGTAGATGGTACTCCAGGACGATTAAACCAAACTAAT 3634
TTTTTAATTAACCGACCTGGATTATTTTATGGACAATGTTCAGAAATTTGTGGAGCTAATCACAGTTTTATACCAATTGTAATTGAAAGAATTCCAGTAA 3734
ATTACTTTATCAAATGAATTTCTAATAATGTAAACTCTTCATTagatgactgaaagcaagtaatggtctc 3801
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Fig. 2. Sequence from a region including the genes for cytochrome oxidase subunits I+II and tRNA-leucine from *C. albiceps* collected in Alexandria, Egypt. Numbers correspond to the homologous sequence in *Drosophila yakuba* (Clary and Wolstenholme 1985). Flanking primer sequences are shown in lowercase. Dashes indicate deletions relative to *D. yakuba*.

biceps specimens did not. Two specimens from South Africa, and 1 from each of the other localities were used.

The Indonesian and Vietnamese specimens were each killed with cyanide and preserved on an insect pin. These were used for DNA extraction within 3 mo (Indonesia) or 2 yr (Vietnam) of collecting. The others were killed and preserved in 95% ethanol, and used within ≈1 yr of collecting. Thoracic muscles, and the entire thorax of each pinned specimen, were removed for DNA extraction. The remainder of each specimen has been deposited as a voucher in the Essig Museum of Entomology, University of California at Berkeley.

DNA extraction and sequencing followed the protocols established by Sperling et al. (1994) for blow flies. Sequencing was performed using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA). The primers used are shown in Table 1. The Sequences from the Egyptian *C. albiceps* specimen and the Florida *C. rufifacies* specimen have been deposited with GenBank (accession numbers AF083657, AF083658).

Phylogenetic analysis was performed using PAUP 3.1.1 (Swofford 1993). An exhaustive search was made to find the most parsimonious tree. The calliphorids *Phaenicia sericata* (Meigen) and *Phormia regina* (Meigen) (Sperling et al. 1994) were used as outgroups. Variable nucleotide positions were treated as 4-state unordered characters. Bootstrap analysis with 500 replications was used to estimate the reliability of individual branches.

Results and Discussion

The entire sequence for the Egyptian *C. albiceps* specimen is shown here. Although it was relatively easy to process ethanol preserved material, pinned specimens presented some technical difficulties. For the Indonesian *C. rufifacies*, we were unable to sequence the antisense strands produced using C1-N-2191 and C1-N-2329 (Table 1), each of which was paired with C1-J-1751a. The overlapping and independently produced sense strands provide confirmation of the sequence for this region. The Vietnamese


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                    1 1
                    5 6
                    7 1
                    2 4
alb. Egypt          T A
alb. S. Africa1     . .
alb. S. Africa2     . .
alb. Brazil         . .
ruf. USA-TX         C .
ruf. USA-FL         C .
ruf. Australia      C .
ruf. Fr. Polynesia  C .
ruf. Indonesia      C G
ruf. Vietnam        C G

                    2 2
                    5 5
                    7 8
                    4 C
alb. Egypt          T T
alb. S. Africa1     . .
alb. S. Africa2     . .
alb. Brazil         . .
ruf. USA-TX         C C
ruf. USA-FL         C C
ruf. Australia      C C
ruf. Fr. Polynesia  C C
ruf. Indonesia      C C
ruf. Vietnam
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Fig. 3. All mitochondri
used in Fig. 2. A dot indica

specimen of *C. rufifaci*
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Parsimony analysis r
monophyletic lineage (
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rate of ≈2.3% per mill
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0.8% within, and 2.9–3.
2 and 3). These results
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These results disting
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RFLP diagnostic test
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lor 1993) found an *Ec*
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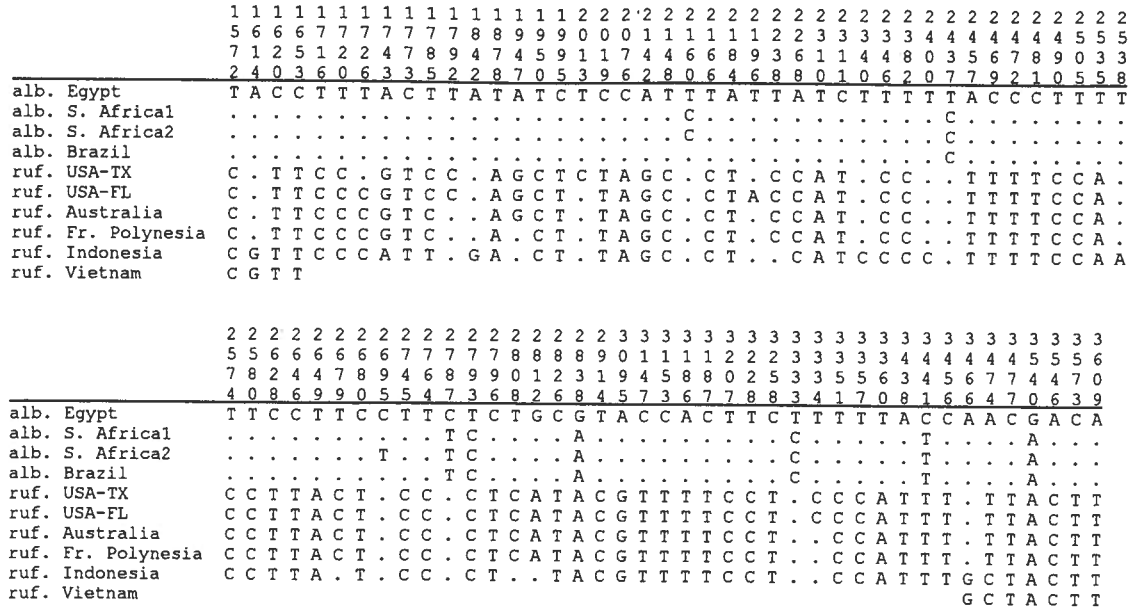

Fig. 3. All mitochondrial DNA sites that varied between specimens of *C. albiceps* and *C. rufifacies*. Numbers are those used in Fig. 2. A dot indicates the same nucleotide found in *C. albiceps* from Egypt.

specimen of *C. rufifacies* generally was difficult to sequence, and it was not included in the parsimony analysis. However, 2 short polymerase chain reaction (PCR) fragments totaling 585 bp from the Vietnamese specimen, produced using primers TY-J-1460/C1-N-1687 and C2-J-3408/TK-N-3782 (Table 1), yielded good sequence data. Furthermore, the Vietnamese and Indonesian sequences were identical and included 3 synapomorphies, and this strongly supports a close relationship between them.

Parsimony analysis revealed that each form was a monophyletic lineage (Fig. 1), and genetic distances suggest that they have been separated for more than a million years. Results from a variety of studies indicated that arthropod mtDNA evolves at an average rate of ≈2.3% per million years (Brower 1994). The *Chrysomya* haplotypes we observed differed by 0.04–0.8% within, and 2.9–3.1% between the 2 forms (Figs. 2 and 3). These results support separate species status for *C. albiceps* and *C. rufifacies*, although this will need to be confirmed by examining specimens from a location where they coexist.

These results distinguish *C. albiceps* from *C. rufifacies* using mtDNA. Sequence data from an unknown specimen could be added to our data set and a phylogenetic analysis performed to find the clade to which the unknown specimen belongs. Alternatively, the sequences presented here may be used to design an RFLP diagnostic test (Sperling et al. 1994). For example, a brief search of our data for diagnostic restriction sites using the program GeneJockeyII (Taylor 1993) found an *Eco*RI site (GAATTC) at position 1,568 of only the *C. albiceps* sequences, and a *Sau*3AI site (GATC) at position 1,618 of only the *C. rufifacies* sequences.

Guimarães et al. (1978, 1979) reported that *C. albiceps* accompanied Portuguese-speaking refugees from Angola to Brazil. Although the sister-group relationship of the South African and Brazilian specimens (Fig. 1) supports this hypothesis, this study did not include (and was not intended to include) a large enough sample of localities to identify the exact source of New World *Chrysomya* populations. However, the topology of the tree in Fig. 1 records a general historical pattern of movement by *C. albiceps* and *C. rufifacies*. The basal positions of the Egyptian *C. albiceps* and Indonesian (and probably the Vietnamese) *C. rufifacies* specimens suggest the outward spread of both species around the globe from a central location in the Old World tropics, most likely from near Pakistan where their historical ranges may overlap (Senior White et al. 1940).

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