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Research Article

Phylogenetic Relationship of the Longhorn Grasshopper *Ruspolia differens* Serville (Orthoptera: Tettigoniidae) from Northwest Tanzania Based on 18S Ribosomal Nuclear Sequences

Nicodemus D. Matojo[1] and Keneth M. Hosea[2]

[1] *Department of Life Sciences, Mkwawa University College of Education (UDSM), P.O. Box 2513, Iringa, Tanzania*
[2] *Department of Molecular Biology and Biotechnology, University of Dar es Salaam, Dar es Salaam, Tanzania*

Correspondence should be addressed to Nicodemus D. Matojo; matojodn@yahoo.com

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Previously, the biology of the longhorn grasshopper *Ruspolia differens* Serville (Orthoptera: Tettigoniidae) from northwest Tanzania was mainly inferred based on the morphological and behavioural characters with which its taxonomic status was delineated. The present study complements the previous analysis by examining the phylogenetic relationship of this insect based on the nuclear ribosomal molecular evidence. In the approach, the 18S rDNA of this insect was extracted, amplified, sequenced, and aligned, and the resultant data were used to reconstruct and analyze the phylogeny of this insect based on the catalogued data.

1. Introduction

The longhorn grasshoppers belonging to the family Tettigoniidae, suborder Ensifera, and order Orthoptera are highly diverse insects with complex evolutionary histories [1, 2]. The family has more than 6,400 species characterized by their long filiform antennae (which may exceed the body length), strong hind limbs, powerful chewing mouthparts, four tarsal segments, male tegminal stridulatory organs, and front tibial tympanum [3, 4]. The genus *Ruspolia* is a group of large, elongate, cone-headed tettigoniids with yellow jaw base [5–8]. These insects usually occur in the grassland and open bushveld; they are active at night, mainly feed on flowers and seeds of cereals especially the millet and maize which they crack using their powerful jaws; males usually produce a very loud continuous hissing call for up to 5 minutes; nymphs hatch in 1-2 months; and they reach adult maturity in 2-3 months [9–12]. Many *Ruspolia* spp. have no apparent diagnostic features, and thus their taxonomy largely requires molecular evidence. However, the relationships among this genus have seldom been inferred based on molecular data [13, 14].

The 18S rDNA is among the most widely used molecular components in phylogenetic analysis of insects [14, 15]. Flook and Rowell [16] and Flook et al. [2] established the phylogenetic relationships among various Orthoptera groups. Liu et al. [17] described the diversity existing among members of the family Acrididae (short-horned grasshoppers). Pratt et al. [18] established the diversity among members of the families Helicidae and Rhaphidophoridae in New Zealand and Australia, whereas Robillard and Desutter-Grandcolas [19] and Danley et al. [20] inferred the phylogenetic relationship of the family Gryllidae (crickets). Molecular data of various *Ruspolia* spp. have been reported from different parts of the world, including Asia, Europe, and Australia [2, 18, 20]; however, there are only scanty published data from east Africa, in which there is an immense abundance of *Ruspolia differens,* a delicacy tettigoniid species widely known as "*senene*" by its Kiswahili name [6, 21].

The majority of the previous works on *Ruspolia* spp. have analyzed various rDNA sequences of 12S and 16S subunits separately without any support from 18S rDNA data and thus no collective results [2, 18]. More recently, Hemp et al. [22] reported the partial sequence of 16S rRNA gene of isolate

M32 of *R. differens* (Accession number FM882032); however, the existing molecular records are insufficient, as they do not convey comprehensive phylogenetic relationship of this species. In this work, the phylogenetic relationship of *R. differens* from Tanzania is analysed critically based on 18S rDNA nuclear sequences.

2. Materials and Methods

R. differens is a swarming insect with the tendency to shift continually within the geographical locality of its subpopulation that can cover a broad range such as Lake Victoria zone [5, 6]. In this study, a total of 90 fresh individuals (adults) of this species were sampled randomly for molecular study. These insects were identified by their key morphological and swarming characters following the descriptions of Bailey [5]. They were collected live from different bushes in Bukoba Rural district (northwest Tanzania) during swarms of April 2009. Immediately after collection, the specimens were rinsed, sacrificed, and preserved by submerging them in 70% ethanol until analysis.

DNA extraction and PCR were conducted at the laboratory of the Department of Molecular Biology and Biotechnology (MBB) of the University of Dar es Salaam, based on the standard protocol [23]. Presence of DNA was checked on 1.5% agarose gel electrophoresis, and viewing was done by using UV fluorescence. A pair of oligonucleotide primers used in this study was adopted from Flook et al. [2]. Each primer contained 21 nucleotide bases for each of the forward and reverse sequences. The expected sizes of PCR product were 826 bp, and the adopted primer sequences were

18S Fwd: GACGAAAAATAACGATACGGG

18S Rev: CTCAATCTGTCAATCCTTCCG

Both primers were ordered in South Africa from a commercial facility, namely, INQABA Biotechnological Industries Pty Ltd. They were shipped under lyophilized state, and their sequences were defined from end $5'$ to $3'$ for convenient handling. Just after collection, each primer was diluted in fresh TE buffer to 200 pmol/μL stock solution. Prior to PCR, the stock solution was diluted to 20 pmol/μL working solution ready for use. All primer solutions were kept under -4°C until PCR.

PCR was conducted in 25 μL volume containing (i) 22 μL mixture of distilled water and puReTaq "ready-to-go" beads (Lot 27-9557-01, GE Healthcare UK Limited) composed of 0.2 mM of dNTP, 0.33 μL Biotaq DNA polymerase, 1.5 μL 10x NH_4 reaction buffer, and 1.5 mM $MgCl_2$, (ii) 1 μL forward primer, (iii) 1 μL reverse primer, and (iv) 1 μL DNA template that was diluted in sterilized distilled water to 10-fold.

PCR reactions were performed in a Veriti Thermal Well Cycler. Amplifications were done based on the following procedure: an initial denaturation of 2 min at 94°C, 30 s at 94°C; annealing of 30 s at 49°C, 35 cycles; extension of 1 min 30 s at 72°C; and a final extension at 72°C for 3 min [18]. PCR products were checked on electrophoresis using 1% agarose gel with ethidium bromide staining, and the results were viewed on UV fluorescence. The PCR products and the respective primers were kept under -20°C and transferred to INQABA (South Africa) where sequencing was conducted.

Prior to sequencing, the PCR products were purified using a QIAquick (QIAGEN) kit. The PCR-amplified DNA fragments were separated by electrophoresis on 1% agarose. A wide-range molecular weight DNA marker (100-bp ladder of 100–5000 bp) was used on each gel as the standard (INQABA Biotechnological Industries Pty Ltd). Gels were stained using ethidium bromide (0.1 μg/mL) for 2 hours. Sequencing was done in an ABI PRISM 3700 DNA analyser using a Big Dye Deoxy Terminator cycle-sequencing kit as per manufacturer's guidelines (Applied Biosystems Inc.).

The reverse 18S rDNA sequence (partial) of *R. differens* was chosen for molecular analysis, since it was clearer. The sequence was aligned into FASTA using CLC Workbench [24], and it was "reverse complemented" using MEGA5 molecular software package (Molecular Evolutionary Genetics Analysis) ready for BLAST search (Basic Local Alignment Search Tool) [17, 25]. The BLAST search was performed online in MEGA5 against the NCBI (National Centre of Biotechnology Information) database where various complete, nearly complete, and partial sequences were widely deposited [2, 16]. For phylogenetic analysis, the various ribosomal 18S nuclear sequences matching with that of *R. differens* were first reverted to FASTA format acceptable by ClustalW alignment. The ClustalW alignment was performed in MEGA5 [26].

A section of the *R. differens* rDNA sequence aligning with the dataset of significant BLAST sequences comprised 480 bp. The resultant combined dataset of the aligned sequences was selected and entered into phylogenetic analysis. Phylogenetic relationship between *R. differens* and other tettigoniids was inferred in MEGA5 based on the nuclear 18S rDNA sequence of this insect and those of its relatives that inferred 98-99% homology in the NCBI BLAST. In this case, the Gryllidae sp. of the order Orhoptera (Accession number EU713460) was added as a chosen outgroup [2]. A phylogenetic tree was reconstructed using the Neighbor-Joining method and Maximum Parsimony method [26, 27]. All positions with less than 95% site coverage were eliminated; that is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position [26].

3. Results

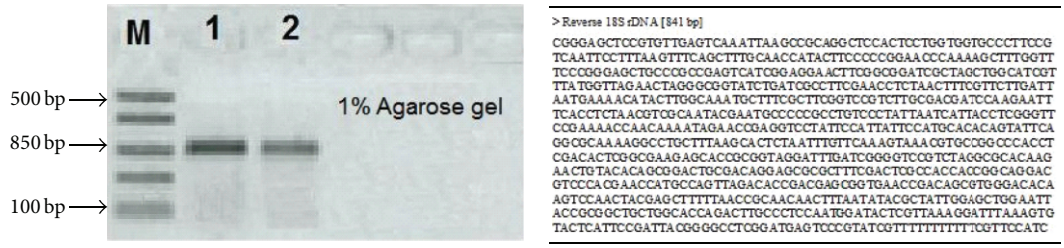
3.1. Sampling and Molecular Methods. The sampling of *R. differens* and subsequent DNA extraction, PCR, and sequencing were successful. PCR products of approximately 850 bp were observed (Figure 1(a)).

3.2. The Nucleotide Sequence. The ascertained partial 18S rDNA reverse sequence of *R. differens* was as represented in Figure 1(b) (length 841 bp).

3.3. BLAST Matches. NCBI BLAST returned a list of previously published ribosomal 18S nuclear sequences significantly aligning with the 18S rDNA sequence of *R. differens* (Table 1). The sequences were reported together with the respective Accession numbers, similarity scores (%), and references.

Table 1: NCBI BLAST sequences matching with 18S rDNA sequence (partial) of *R. differens* from Tanzania.

Accession number	Nearest taxon	Nucleotide sequence (partial)	Similarity score	Reference
Z97582	*Ruspolia nitidula*	18S rRNA	99%	Flook and Rowell [16]
JF792564	*Ruspolia lineosa*	18S rRNA	99%	Wang et al. [28]
JF792563	*Ruspolia dubia*	18S rRNA	99%	Wang et al. [28]
JF792565	*Conocephalus maculatus*	18S rRNA	99%	Wang et al. [28]
JF792566	*Conocephalus gladiatus*	18S rRNA	99%	Wang et al. [28]
JF792574	*Phyllomimus sinicus*	18S rRNA	99%	Wang et al. [28]
JF792573	*Orophyllus montanus*	18S rRNA	99%	Wang et al. [28]
EU713460	*Gryllidae* sp.	18S rRNA	98%	Flook et al. [2]



(a) (b)

Figure 1: Gel photo (a) showing PCR products of partial 18S rDNA reverse sequence (b) of *R. differens* (M represents DNA size marker).

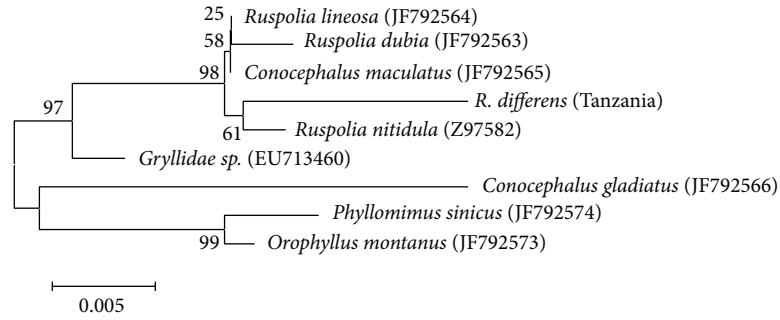


Figure 2: The phylogeny of *R. differens* and relatives inferred from the nuclear ribosomal 18S sequences using the Neighbor-Joining method. The tree is drawn to scale, and the branch lengths are in the same units as the evolutionary distances. Bootstraps (1050 replicates) are shown next to the branches. The optimal tree (length = 0.06974) is shown, with a total of 483 positions in the final dataset.

3.4. Phylogeny. Figures 2 and 3 present a phylogram of *R. differens* and relatives based on the ribosomal 18S nuclear sequences inferred based on Neighbor-Joining and Maximum Parsimony methods, respectively. In the tree, *R. differens* is clustered together with all other *Ruspolia* spp. included in the analysis, forming a distinct clade. The closest genus shows to be *Conocephalus* (under 98% bootstrap), whereas the closest relative of *R. differens* indicates to be *R. nitidula* (within 61% bootstrap).

3.5. Relationship Patterns. Both of the resultant phylograms (Figures 2 and 3) have indicated that all of the comparable species are closely related to *R. differens* with the exception of Gryllidae sp. that was a chosen outgroup. However, *R. nitidula* demonstrates to be the closest species followed by *R. lineosa* and *R. dubia*. The closest genera to the genus *Ruspolia* indicates to be *Conocephalus*.

For comparison and verification of the relationship inferred by the nuclear ribosomal 18S dataset, the study further presents the phylogeny resulting from NCBI BLAST of 16S rRNA partial sequence of *R. differens* (Accession number FM882032) by Hemp et al. [22]. All sequences (Figure 4) inferring the homologies of 98-99% are included in the phylogeny, and there is a sequence of *Gryllus campestris* (Accession number Z93318) as a chosen outgroup from a distant family, namely, Gryllidae also of the order Orthoptera. Figure 4 presents the resultant phylogeny tree generated from this dataset. Likewise, *R. differens* has clustered closely with

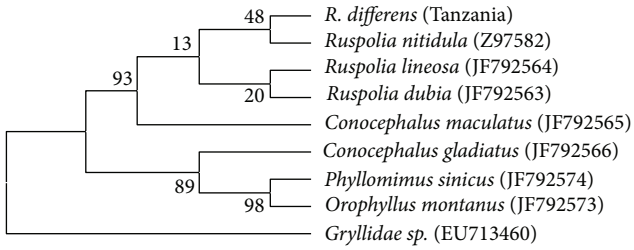


Figure 3: The phylogeny of *R. differens* and relatives inferred from the nuclear ribosomal 18S sequences using Maximum Parsimony method. Bootstraps (1050 replicates) are shown next to the branches. The first tree of 21 most parsimonious trees (length = 47) is shown, and there were a total of 445 positions in the final dataset.

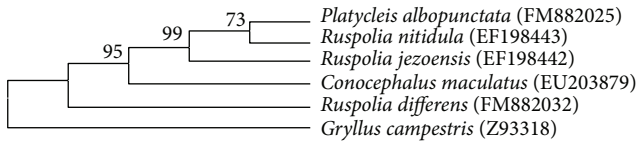


Figure 4: The phylogeny of *R. differens* and relatives inferred from the related catalogued nuclear ribosomal 16S sequences using Maximum Parsimony method. The most parsimonious tree (length = 424) is shown, and there were a total of 455 positions in the final dataset [26].

other *Ruspolia* spp. forming a distinct clade similar to that inferred in the previous section by the nuclear ribosomal 18S molecular data. The closest genus indicates to be *Conocephalus,* whereas the closest species include *R. nitidula*.

4. Discussion

Prior to this study, the 18S rDNA sequence of *R. differens* from Bukoba Tanzania was unknown, and thus it was not catalogued in any genebank. In addition, there was no detailed phylogeny reconstructed deliberately to define the relationship of this insect with other tettigoniids based on the nuclear genetic subunits. The present study has successfully used the molecular tool to isolate, sequence, and align the 18S rDNA sequence of *R. differens* based on standard protocols. Then the sequence was used to analyze the phylogenetic relationship of this insect and its relatives.

The sampling of *R. differens* and subsequent molecular methods applied in this study, including DNA extraction, primer designing, PCR, sequencing, and phylogenetic analysis, were in line with those used by various other workers as they have yielded comprehensive and workable data and relationships. The intense appearance of the DNA lanes (Figure 1) indicates that the preparations were of good quality. The gel lanes have only slight smears indicating that the DNA template used was quite large and unbroken. Prospective workers can adopt similar methodology to conduct related molecular studies.

The phylogeny of *R. differens* reconstructed in this study based on 18S rDNA sequence with the support from a phylogeny reconstructed using the catalogued 16S rRNA sequence [22] provides a useful picture on the phylogenetic relationship of this insect. In both cases (Figures 2–4), *R. differens* has demonstrated to be a true member of the genus *Ruspolia*. Its closest relative indicates to be *R. nitidula* occurring in the Palearctic region. The findings agree well with various other workers that *R. nitidula* is the closest relative and a sibling of *R. differens* [5, 6]. Other sister species include (in brackets being their chief geographical distribution) *R. jezoensis* (Asia-temperate), *R. dubia* (Asia), *R. lineosa* (Asia), *C. maculatus* (Europe), and *P. albopunctata* (Tropical Africa, living sympatrically with *R. differens*). All these insects belong to the subfamily Copiphorinae of the family Tettigoniidae.

However, there is a clear disagreement between the present findings and those of a few other workers who diagnosed *R. differens* as *R. nitidula* [21, 29, 30]. The present findings have clearly demonstrated that these are two different species with different molecular affiliations. The shortcomings might have been contributed by the fact that, prior to this study, there were no reliable molecular data to discriminate *R. differens* from its relatives.

5. Conclusions

We conclude that the molecular information available in the partial 18S rDNA sequence of *R. differens* concur well with those of the catalogued sequences upon which the various phylogenetic relationships of this insect and other longhorn grasshoppers (family Tettigoniidae) can be clearly inferred, and thus it is useful to incorporate these sequences in the standard molecular database for reference by subsequent workers; the relationships inferred by the 18S rDNA sequence of *R. differens* are in line with those generated from the catalogued 16S rRNA sequence by Hemp et al. [22] in which it was clearly verified that the closest genera to the genus *Ruspolia* is *Conocephalus* and the closest relative to *R. differens* is *R. nitidula*; the molecular phylogeny of *Ruspolia* spp. and relatives is a true function of their geographical affiliations, whereas *R. differens* chiefly occurs in the African Tropics, its closest relative, that is, *R. nitidula,* is a Palearctic species occurring in North Africa, Europe, and Asia, while the rest of the other close *Ruspolia* spp. inferred, that is, *R. dubia, R. lineosa,* and *R. jezoensis,* are typically European and Asian species; the worldwide distribution of the Ruspolia coneheads, which is well bridged by *R. nitidula,* clearly indicates that the genus *Ruspolia* originated in the African Tropics before radiating to northern Africa, Europe, and Asia; the 18S rDNA sequence of *R. differens* and the inferred phylogenetic relationship appear to be a useful tool for further studies on the ancestry of the tettigoniids, related taxa, and some complex phenomena such as swarming behaviour, colour polymorphism, and unique anatomy, the genes of which remain to be investigated; and *R. differens* and its sequence are of an outstanding value in research as they are capable to reveal various unresolved phylogenetic relationships. Further supportive molecular attention on *R. differens* is required, including analysis of the genes for the many unique characters of this insect.

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