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Amplification of *COI-II, ND II & ITSI-II* by Polymerase Chain Reaction: Prospects for DNA Barcoding of Nigerian *Drosophila* Populations

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ABSTRACT: Populations of Nigerian *Drosophila* are hardly distinguishable in morphology but reproductively isolated. Spectrophotometric analysis of genomic DNA of the fruitflies reveals that the DNA extraction protocol used yielded genomic DNA good enough for polymerase chain reaction, confirming claims that polymerase chain reaction is possible even when amount of template DNA varies considerably. The mitochondrial cytochrome oxidase subunits I and II, nicotinamide adenine dinucleotide dehydrogenase subunit II, and the nuclear internal transcribed spacer regions I and II are highly conserved regions in *Drosophila*. Successful amplification of these regions suggests that they are promising regions for DNA barcoding and molecular characterization of local *Drosophila* species. Further molecular and DNA barcoding studies targeted at local *Drosophila* species in Nigeria is desirable.

Keywords: *Nigeria, Drosophila, ND2, COI, COII, ITSI, ITSII*

Introduction

Drosophila are small insects which belong to the order *Diptera*. Species of *Drosophila* are used as model organism for researches in genetics, developmental biology, and evolution (O'Grady & Markow, 2009). Polymerase chain reaction (PCR) is a biochemical technique in molecular biology that is used to amplify deoxyribonucleic acid (DNA) in order to generate several copies of a particular DNA sequence. It is now a common technique used in biological research for a variety of applications including DNA-based phylogeny (Saiki *et al*., 1988). PCR may be used to isolate DNA fragments from genomic DNA by selective amplification of a specific region of DNA before being examined for polymorphism. This helps to augment other molecular methods which require larger amounts of DNA, representing a specific DNA region. PCR is also applicable in DNA sequencing to determine unknown PCR-amplified sequences. PCR supplies these molecular techniques with high amounts of pure DNA, enabling analysis of DNA samples even from minute amounts of starting material.

The DNA extraction procedure required for PCR process should be such that will minimise impurities otherwise, the PCR process will fail. Colton & Clark (2001) indicated that successful PCR is possible when the amount of template DNA used for amplification varies widely, even up to fifteen-fold and that quantitation of DNA is not necessary.

Morphological methods Only have proved inadequate for characterization, identification and phylogenetic analysis of organisms. Molecular methods are now being employed to improve the reliability of insect characterization. Studies (Li *et al.*, 2011; Cameron *et al.*, 2006; Young & Coleman, 2004) have successfully used PCR-based molecular methods in the characterization, identification and taxonomy of *Drosophila* from different parts of the world. Recently, DNA barcoding has been proposed as a fast and reliable way of characterizing organisms. DNA barcoding involves generating barcodes for DNA sequence of standard gene regions and comparing these barcodes of different organisms.

For DNA barcoding, standard gene regions such as CO I, CO II of the mitochondria; and nuclear ITS I and ITS II have been proposed. The hyper-variable non-coding control regions of the mitochondrial genome (including CO I, CO II and ND II) and the nuclear ribosomal DNA (ITS I and ITS II) - preferred for its low level intraspecies sequence variation and higher degree of interspecies differences - are highly conserved regions in *Drosophilids*. Song *et al.* (2008) suggested that the rDNA ITS2 region is a good phylogenetic marker at the species or genus levels due to the ease of PCR amplification and being a relatively short region with high information content.

Studies (Stage & Eickbush, 2007; Schlotterer *et al.*, 1994) comparing sequence variation of rDNA in Drosophilids demonstrated that the 3' end of ITS I and 5' end of ITS II were highly conserved, showing sequence identity across all species. Stage and Eickbush (2007) further suggested that sequence conservation in these two regions was a result of secondary structures needed for processing the primary RNA transcript. The ITS II secondary structure has been shown to be a feature of major animal groups including Drosophilids, making the region ideal for reconstructing evolutionary relationships at the species and genera levels (Schlotterer *et al.*, 1994; Young & Coleman, 2004).

The first subunit of the cytochrome oxidase (CO) gene, corresponding to nucleotides 1490-2198 of the *Drosophila yakuba* sequence has been identified as a region of scientific interest for DNA barcoding (Hebert *et al*., 2003; Nelson *et al*., 2007). Wallman *et al.* (2005) combined the gene sequences of CO I and CO II with ND4 and ND4L in an attempt to clarify evolutionary relationship and correctly identify species of *Chrysomya* (blowflies). Most research support the use of cytochrome oxidase subunits I and II in molecular identification, however, some have reported challenges caused by the bacterium *Wolbachia*, a maternally transmitted endosymbiont of insects that cause some reproductive alterations in the insects (Bruhn, 2011).

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Most studies on Nigerian *Drosophila* species has been largely on genetic load and lethal allelism (Adekoya, 2001 and Adekoya and Williams, 2001), Inversion polymorphisms (Adefenwa *et al.*, 2010). Perhaps, little or no attempt has been made to amplify any of the standard regions in Nigerian *Drosophila* populations. Considering the prospect of DNA barcoding, a preliminary study is needed to ascertain that genetic variability exist in the standard gene regions (ITS I, 1TS II, CO I, CO II and ND II) of Nigerian *Drosophila* populations.

Materials and Methods

Wild *Drosophila* samples were collected from four locations in Nigeria: EtiOsa– 6º26'48.85"N, 3º28'37.12"E; Akoka– 6º30'57.88"N, 3º23'49.56"E; Abeokuta – 7º07'54.57"N, 3º20'59.31"E; and Ijebu Ode - 6^{O}49'18.00"N, 3^{O}56'23.22"E) between May and July.
Genomic DNA of flies was extracted using modified Goldenberger *et al.* (1995) protocol, making use of 10% sodium dodecyl sulphate (SDS) and Proteinase K followed by 24hrs of incubation at 37ºC. *Drosophila* flies were anaesthetised with diethyl ether and 0.5g of each sample was ground in 900µl of 1 X TNE buffer using pestle and mortar. The homogenate was transferred into 1.5ml eppendorf tube and 100µl of 10% Sodium dodecyl sulphate (SDS) was added to it and then shaken vigorously. 5µl of Proteinase K was added to each sample and inverted few times. The samples were later incubated at 37ºC in a water bath for 24 hours. 150µl of supersaturated 5M NaCl (cold) was added to each sample and was later kept ice cold for 30 minutes.
The samples were then spun at 14,000 rpm for 10 minutes after which the supernatant was transferred into another set of 1.5ml eppendorf tubes. 1,000µl of cold ethanol was added to each tube and then centrifuged at 8,000rpm for 10 minutes. Supernatant was discarded and DNA pellets were air dried at room temperature for 24 hours and then kept at -20^{O}C prior to quantitation, electrophoresis and PCR.
The concentration of extracted DNA was determined by using a NANODROP 1000 spectrophotometer. It was first initialized with distilled water. 5µl of each DNA sample was loaded in the spectrophotometer which was connected to a computer system, and readings were taken electronically. Concentration of the DNA was determined in ng/µl. Spectrophotometry was used to assess DNA purity.Quality of extracted genomic DNA was also checked on 1% agarose gel electrophoresis. Photographs were taken under a shortwave UV light illuminator.
The primers (Table 1) were spun down at 14,000rpm for 1 minute and reconstituted with ultra-pure distilled water as described by the primer synthesis report. They were immediately kept at -20^{O}C. PCR tubes were labelled appropriately. The PCR master mix, containing every other ingredient except the DNA sample was prepared in a 1.5ml eppendorf tube on ice. This was then vortexed, centrifuged for few seconds and kept ice cold. 17µl of master mix was aliquot to each individual reaction tube before adding 3µl of DNA samples. Reaction tubes were loaded into PCR machine and the appropriate programs for each primer were set.

Table 1: Target regions and primer sequences used for this study

Target Region	Primer sequence (5'-3')	Length (bp)
Mitochondrial loci		
ND2	AAGCTACTGGGTTCATACC ATATTTACAGCTTTGAAGG	926
COI-COII	ATACCTCGACG(AT)TATTGA GTTTAAGAAACCAGTACTTG	842
Nuclear locus		
ITS1-ITS2	TCCGTAGGTGAACCTGCGG GTTAGTTTCTTTTCCTC	650
Total		2418

The target regions were amplified on an iCycler Thermal Cycler (Bio-Rad). The PCR cycle program comprised of a 5 min of predenaturation at 94^{O}C, 35 cycles of amplification (1 min of denaturing at 94^{O}C; 1min of annealing at 53^{O}C for *COI-II* and *ND2*, 56^{O}C for ITS1- ITS2, 1 min of extension at 72^{O}C), and final extension at 72^{O}C for 5min. PCR products were verified using 1% agarose gel electrophoresis in a 1 X Tris-Borate-EDTA (TBE) buffer. Agarose was prepared by boiling 1g of the agarose powder in 100ml of 1 X TBE buffer. After boiling the solution was allowed to cool before 40µl of ethidium bromide was added to it. This was then poured into the gel casting tray with its combs placed across its rim to form wells. The gel was allowed to set for 20 minutes and the combs were removed. 5µl of amplicons from each sample were loaded into the well. A DNA molecular weight marker was also loaded into one of the wells. The gel was thereafter electrophoresed in a horizontal tank at a constant voltage of 50 Volts for about 1 hour. After completion of electrophoresis, bands of DNA were viewed as florescence of bound ethidium bromide under a shortwave ultraviolet light illuminator and photographs were taken.

Results

SDS-Proteinase K DNA extraction protocol gave high yield of DNA with mean value of 1322.61ng/µl and standard deviation value of 946.84 for all the locations (Table 2).

Table 2 Summary statistics for DNA concentration and purity for all samples

Sampling site	DNA concentration (ng/µl) Mean ± SEM (n=6)	Purity (OD_{260nm}/OD_{280nm}) Mean ± SEM(n=6)
Eti-Osa	1299.93 ± 75.21	1.87 ± 0.03
Akoka	1442.95 ± 20.15	1.90 ± 0.05
Abeokuta	1912.87 ± 46.17	2.03 ± 0.90
Ijebu- Ode	634.67 ± 13.55	1.95 ± 0.01

The mean concentration of DNA extracted from each of the 4 locations is shown in Fig. 1 while Fig. 2 shows the mean values for purity of DNA of the 4 locations. Mean purity of all extracted DNA is 1.94±0.04.

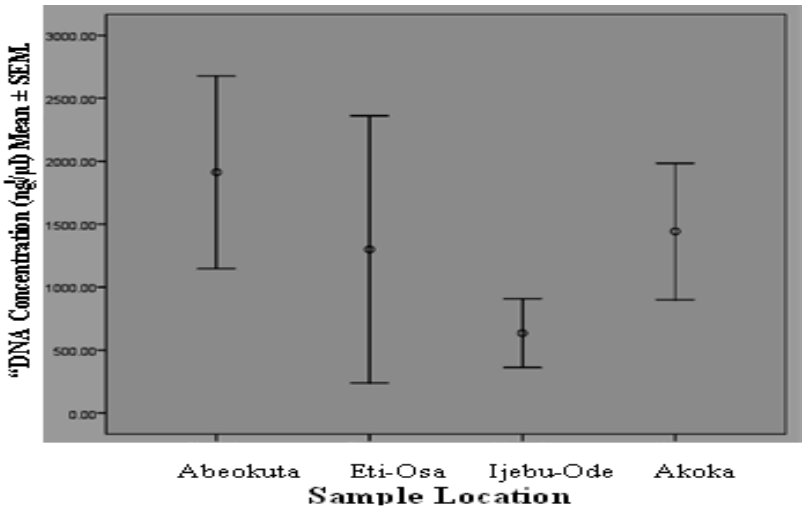


Fig. 1: Mean concentration of DNA extracted from four *Drosophila* populations.

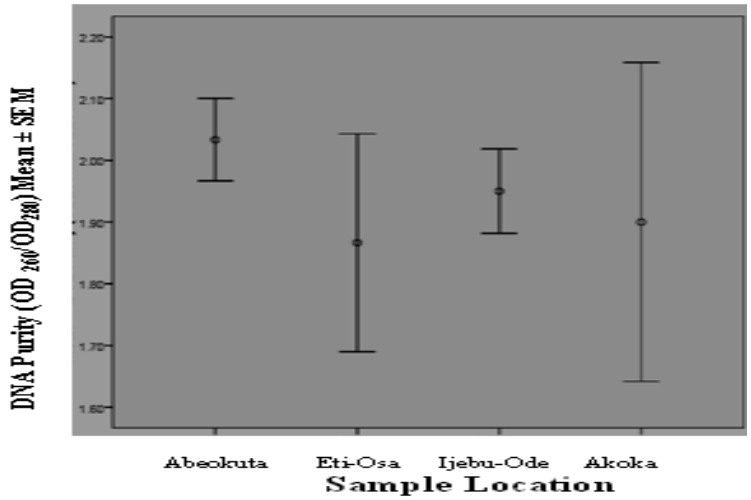
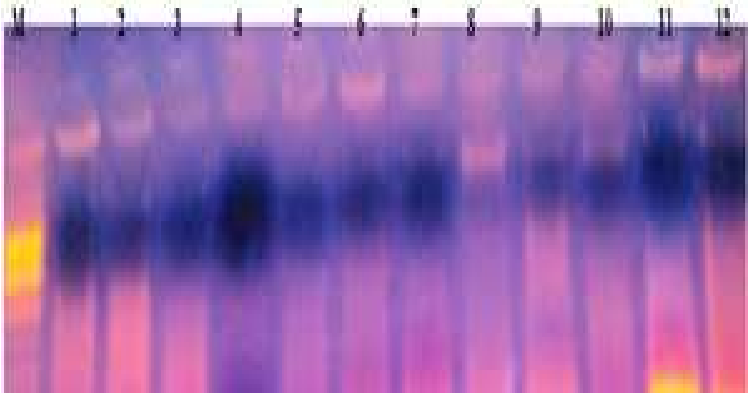


Fig. 2: Mean purity of DNA extracted from four *Drosophila* populations

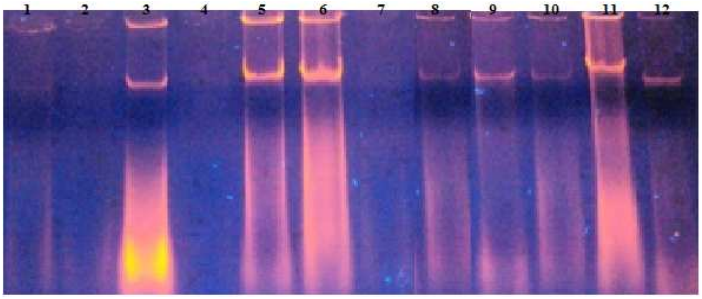
Fig. 3 shows the gel photograph of genomic DNA checked on 1% agarose gel electrophoresis. All the lanes showed bands confirming the presence of genomic DNA for the four locations and they were all observed to be less than 1kb.



Legend
M= 1Kb ladder
1-3 = Abeokuta
4-6 = Etiosa
7-9 = 1/Ode
10-12 =
UNILAG

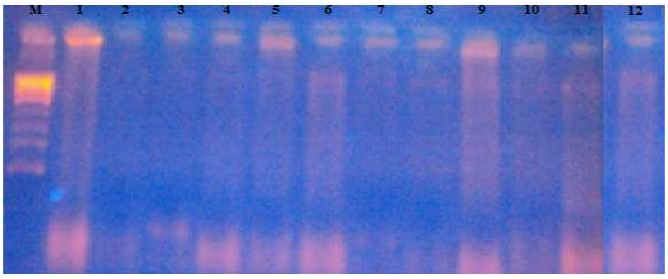
Fig. 3: Agarose gel photograph of genomic DNA extracted from *Drosophila* populations collected from four selected locations in Lagos and Ogun states.

PCR was done separately using primers for each of the three regions (ITS I-ITS II, CO I-CO II and ND II) and thereafter, 1% agarose gel electrophoresis was used to verify the PCR products (amplicons). The electrophoresis gel photographs are displayed in Figs 4-6.



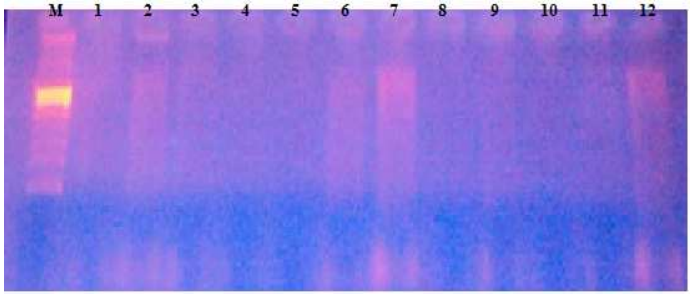
Legend
M= 1Kb ladder
1-3 = Abeokuta
4-6 = Etiosa
7-9 = 1Ijebu Ode
10-12 = UNILAG

Fig 4: Agarose gel photograph of *COI-COII* amplicons of DNA extracted from the four *Drosophila* populations



Legend
M= 1Kb ladder
1-3 = Abeokuta
4-6 = Etiosa
7-9 = 1Ijebu Ode
10-12 = UNILAG

Fig. 5: Agarose gel photograph of *ND2* amplicons of DNA extracted from the four *Drosophila* populations



Legend
M= 1Kb ladder
1-3 = Abeokuta
4-6 = Etiosa
7-9 = 1Ijebu Ode
10-12 = UNILAG

Fig. 6: Agarose gel photograph of *ITSI-ITSII* amplicons of DNA extracted from the four *Drosophila* populations

Discussion

Spectrophotometric analysis (Table 2) shows that the DNA extraction method (SDS-Proteinase K based) used gave a mean yield of ≈1,323 ng/µl and also that the extracted genomic DNA barely has protein impurities with mean purity estimate of 1.94±0.04 for all samples. The above results indicate that most of the extracted DNA is of good quality, with minimal protein impurities (OD_{260nm}/OD_{280nm}) based on the standard purity values.

Fig. 1 shows the level of variation in the template DNA prior to PCR. Despite this variation, amplification of target regions was still possible as observed in Figures 4-6. From this, it could be inferred that the DNA extraction method used (modified Goldenberger *et al.*, 1995) yielded good quality and quantity of genomic DNA sufficient for amplification by polymerase chain reaction (PCR). This result further corroborates the findings of Colton & Clark (2001), indicating that successful PCR is possible when the amount of template DNA used for amplification varies widely, even up to fifteen-fold and that quantitation of DNA is not necessary, a conclusion with practical implications when the amount of a fly sample is limiting.

The electrophotograph for the genomic DNA (Fig. 3) also reveals that all the samples produced visible bands on 1.0% gel electrophoresis. Most of the bands were < 1000bp. Examination of gel electrophotograph of CO I-CO II amplicons (Figure 4) of the genomic DNA of the *Drosophila* samples reveals that most of them have visible bands for the selected gene region. This suggests that CO I-CO II is a promising region that could be used for DNA barcoding and molecular characterization of local *Drosophila* species at both species and genus levels. The result agrees with the findings of Hebert *et al*. (2003) and Nelson *et al.* (2007) when they identified CO I as a region of scientific interest for DNA barcoding.

Most studies support the use of CO I and CO II in molecular identification. However, as Bruhn (2011) reported, the maternally transmitted *Wolbachia* may influence mtDNA variations. Considering this challenge, another mitochondrial region such as *ND2* may

be used to further support the studies. Wallman *et al*. (2005) combined CO I and CO II with ND 4 and ND 4L gene sequences in order to unambiguously identify several *Chrysomya* (blowflies) species. Examination of gel electrophotograph of ND II amplicons (Figure 5) of the genomic DNA of *Drosophila* samples reveals that all the lanes have visible bands for ND II. As shown in earlier studies (Wallman *et al.*, 2005), this finding also supports claims that the ND region, being a mitochondrial region also, could also be used as marker together with CO I for DNA barcoding studies and molecular characterization of *Drosophila*.

Fig. 6 shows the gel electrophotograph of ITS I- ITS II amplicons of the genomic DNA of *Drosophila* samples and reveals that most of the samples have bands for the selected gene region. The above result is an indication that the nuclear gene ITS I- ITS II is also very useful for molecular characterization and DNA barcoding studies in *Drosophila*. In a study, (Li *et al.*, 2011), the authors proposed the use both mitochondrial and nuclear markers for such molecular characterization and DNA barcoding studies. Previous studies (Schlotterer *et al.*, 1994; Young & Coleman, 2004) revealed that ITS region is ideal for reconstructing evolutionary relationships at the species and genera levels. Song *et al.* (2008) suggested that the rDNA ITS2 region is a good phylogenetic marker at the species or genera levels due to the ease of PCR amplification and the high information content of the relatively short region.

Based on the findings of this study that CO I-CO II, ND II and ITS I-ITS II are well conserved in selected local populations of *Drosophila*, it is suggested that further molecular and DNA barcoding studies targeted at local *Drosophila* species in Nigeria be carried out.

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References

Adekoya, K.O. (2001)**:** Second Chromosome viability genetic load and lethal Allelic Frequency in *Drosophila melanogaster* in Ewekoro, Ogun State, Nigeria. *J. Res. Rev. Sci.* **2**: 178 – 184.

Adekoya, K.O and Williams, G.O. (2001)**:** Second Chromosome viability Genetic Load and Lethal Allelic Frequency in *Drosophila melanogaster* in Lagos Nigeria. *J. Sci. Tech. Environ.* **1 (1)** : 77-84.

Adefenwa, M.A., Williams, G.O., Oboh, B.O. and Adekoya, K.O**.** (2010): Inversion polymorphism in two populations of *Drosophila melanogaster* in Nigeria. *J. Sci. Res. Dev.* **12 (1) :** 53-64.

Bruhn, T. (2011): *Sequence and analysis of the mitochondrial DNA control region of nine Australian species of the genus Chrysomya (Diptera: Calliphoridae).* MSc. thesis, University of Wollongong, Australia. 148pp.

Cameron, S., Lambkin, C., Barker, S. and Whiting, M. (2006). A mitochondrial genome phylogeny of Diptera: whole genome sequence data accurately resolverelationships over broad timescales with high precision. *Syst. Entomol.* **32** (1): 40-59.

Colton, L. and Clark, J. B. (2001): Comparison of DNA isolation methods and storageconditions for successful amplification of *Drosophila* genes using PCR. *Drosophila Information Service*. **84**: 180-182.

Goldenberger, D., Perschil, I., Ritzler, M., and Altwegg, M. (1995): A simple "universal" DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. *Genome Res.* **4** (6), 368–370.

Hebert, P. D. N., Cywinska, .A., Ball, .S.B. and deWaard, .J.R. (2003): Biological identifications through DNA barcodes. *Proc. Roy. Soc. B* **270:** 313–321.

Li, Y.F., Wen, S.Y., Kawai, K., Gao, J.J., Hu, Y.G., Segawa, R and Toda, M.J (2011): DNA Barcoding and Molecular Phylogeny of *Drosophila lini* and its sibling Species. *Intl. J. Evol. Biol.* .**2012**: 1-9.

Nelson, L. A., Wallman, J. F. and Dowton, M. (2007):. Using COI barcodes to identify forensically and medically important blowflies. *Med. Vetern. Entomol.*. **21**: 44-52.

O'Grady, P.M. and Markow, T.A. (2009):. Phylogenetic taxonomy in *Drosophila*: Problem and Prospects. *Fly. 3: 1-5.*

Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K., and Erlich, H. (1988): Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239** (4839): 487–491.

Schlotterer, C., Hauser, M., von Haeseler, A. and Tautz, D. (1994). Comparative evolutionary analysis of rDNA ITS regions in *Drosophila*. *Mol. Biol. Evol.* **11**(3):5 13-522.

Song, Z., Wang, X., Liang, G. (2008): Species identification of some common necrophagous flies in Guangdong province, southern China based on the rDNAinternal transcribed spacer 2 (ITS2). *Foren. Sci. Intl*.**175** (1):17-22.

Stage, D. E.andEickbush, T.H. (2007). Sequence variation within the rRNA gene lociof12 Drosophila species.*Genome Res.* **17**: 1888–1897.

Wallman, J. F., Leyss, R. and Hogendoorn, K. (2005): Molecular systematics ofAustralian carrion-breeding blowflies (Diptera: Calliphoridae) based onmitochondrial DNA. *Invert. System.* **19**: 1-15.

Young, I. and Coleman, A.W. (2004): The advantages of the ITS2 region of the nuclearrDNA cistron for analysis of phylogenetic relationships of insects: a Drosophilaexample. *Mol. Phylog. Evol.* **30:** 236–242.