A study on prevalence and molecular characterization of trypanosomal species infecting equines in Lahore region, Pakistan

Naveed Sabir¹ · Zafar Iqbal Chaudhry² · Asim Aslam² · Khushi Muhammad² · Muhammad Shahid¹ · Abid Hussain³ · Shahzad Akbar Khan³ · Ishtiaq Ahmad²

Received: 25 September 2017 / Accepted: 6 December 2017 / Published online: 12 December 2017
© Indian Society for Parasitology 2017

Abstract Trypanosomiasis is an important protozoal disease with a diverse range of susceptible host including human. In the current study, molecular characterization of prevalent species was done through a pan-trypanosome polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). A total of three hundred (n = 300) equines including horses, donkeys and mules (100 each) were randomly selected and the equine blood samples were subjected to screening for trypanosomes through microhaematocrit centrifuge technique (MHCT), conventional PCR, semi-nested PCR and RFLP. Overall prevalence of trypanosomal species was 8% (24/300) as revealed by MHCT and species wise prevalence in horses, donkeys and mules was 4.33% (13/300), 1.33% (4/300) and 2.33% (7/300), respectively. Conventional and semi-nested PCR depicted an overall prevalence of 21% (63/300) and species wise prevalence in horses, donkeys and mules was 12% (36/300), 3.67% (11/300) and 5.33% (16/300), respectively. RFLP analysis of the semi-nested products, using *Msp1* and *Eco571* enzymes, negated the presence of *T. congolense*, *T. brucei*, *T. vivax*, *T. theileri*, and *T. vivax* in the positive samples and revealed that the animals might be suffering from *T. evansi* infection as the enzymes used were not able to detect this species. This

hypothesis was further confirmed by using *T. evansi* specific primers which depicted all of the 63 samples were positive for *T. evansi*. It is inferred that *T. evansi* is the major *trypanosome* species prevalent in equines. Furthermore, PCR is more sensitive as compared to microscopic examination and the pan-trypanosome PCR–RFLP assay is suitable for carrying out laboratory diagnosis of field samples and epidemiological studies. Further studies on the possibilities of use of other restriction enzymes may help to improve the species specificity of the assay.

Keywords Prevalence · Molecular characterization · Trypanosomiasis · Equines · MHCT · PCR–RFLP

Introduction

Trypanosomiasis, also known as trypanosomosis, is a protozoal infection causing diverse type of diseases in a variety of mammalian species including human. The severity of the disease varies among animal species. According to economic survey of Pakistan, the population of horses, donkeys and mules is 5.00, 0.20 and 0.40 million numbers, respectively (Anonymous 2016). Equines play an important role in the agricultural economy of the areas where poor infrastructure and an uneven topography makes transportation by vehicle inaccessible. In these remote areas and even in some cities, equines are used for transportation, riding, carting, and threshing farm cultivation among other things. These animals are susceptible to a variety of trypanosome species including *T. evansi*, *T. congolense*, *T. vivax*, *T. brucei* and *T. equiperdum*. The disease has received deep consideration and attention due to its devastating effects on the health of working equines. Trypanosomal infestation makes an animal unable to

✉ Naveed Sabir
drnaveed_vet@yahoo.com

¹ College of Veterinary Medicine, China Agricultural University, Beijing 100193, China

² Faculty of Veterinary Science, University of Veterinary and Animal Sciences (UVAS), Out Fall Road, Lahore 54000, Pakistan

³ Faculty of Veterinary and Animal Science, The University of Poonch Rawalakot, Rawalakot 12350, Pakistan

accomplish its duty and gradually leads the victim towards death. In equines, the disease is acute and fatal while in camels it is chronic in nature resulting in progressive weakness and emaciation of the animals (Gill 1991). Collection of factual data about the epidemiology of disease is essential for the development of an effective treatment and control strategy to protect equines and livestock against trypanosomiasis. Mostly, the disease is diagnosed by clinical signs, microscopic examination of the blood smear or serological methods. However, these methods are not effective and show unsatisfactory results especially in cases of low parasitaemia (Nantulya 1990; Olaho-Mukani et al. 1993).

The polymerase chain reaction (PCR) permits identification of parasites at levels far below the detection limit of the commonly used parasitological techniques. Thus, PCR assays for trypanosome detection have been developed (Desquesnes and Tresse 1996; de Almeida et al. 1997) using species specific primers. But use of species specific primers implies several PCR tests to be conducted to ascertain the presence or absence of a certain species or even in case of mixed infections. This leads to an increase in cost and time required for diagnosis. Efforts to use multiplex PCR have been discouraged due to lower sensitivity and specificity. The development of a universal single PCR based on ribosomal genes (McLaughlin et al. 1996) and its further evaluation (Desquesnes et al. 2001) has enabled us to detect combined trypanosome infections through a single PCR. Geysen et al. (2003) successfully characterized different trypanosome species infecting cattle by using PCR–RFLP approach. In another study (Njiru et al. 2005), amplification of internal transcribed spacer (ITS1) of rDNA has been evaluated to be used as a universal diagnostic test for all pathogenic trypanosomes of bovines and camels in Africa. Recently, Biryomumaisho et al. (2013) studied the prevalence of different trypanosomal species in cattle, goat and pigs by targeting internal transcribed spacer 1 (ITS1) of ribosomal DNA through PCR.

In Pakistan, variable prevalence of trypanosomiasis in horses has been reported by different authors by using conventional and molecular techniques through species specific primers (Anwar and Mohammed 1986; Bano and Jan 1986; Waheed et al. 1998; Muieed et al. 2010; Aslam et al. 2010; Nadeem et al. 2011) but there is still lack of data about the prevalence of disease in donkeys and mules. Therefore, the current project was aimed to characterize different trypanosome species infecting equines by targeting 18S ribosomal sub-unit sequence (Ssu-rDNA) which is highly conserved and is a multi-copy locus sequence.

Materials and methods

Selection of animals and collection of blood samples

A total of three hundred (300) equines including horses, donkeys and mules, irrespective of sex and age, were selected for sample collection from outdoor hospital, Society for the Prevention of Cruelty to Animals (SPCA), and Brooke's Hospitals of Lahore and adjacent areas. Blood samples (5 ml) were collected aseptically from jugular vein in EDTA coated tubes.

Microhaematocrit centrifugation technique (MHCT)

The samples were immediately processed for microscopic examination of buffy coat as described earlier (Benjamin 1986). Briefly, after centrifugation of capillary tube (75 × 1.5) at 5000 rpm for 5 min, the capillary tubes were broken 1 mm above the buffy-coat layer. The contents of this piece were partially expelled onto a slide and stained with Giemsa stain. Blood smears were subjected to microscopic examination (100x) for the presence or absence of trypanosomes. The remaining part of the samples was stored at − 80 °C to be used for DNA extraction and further processing.

DNA extraction

DNA from the blood samples was extracted according to the manufacturer's instructions (Genomic DNA Purification Kit, Fermentas Inc.). Briefly, 200 µl blood samples was lysed with 400 µl of DNAs free water, spun down at 10,000 rpm for 2 min and the pellet was suspended in 200 µl of TE buffer. 400 µl lysis solutions was added and incubated at 65 °C for 5 min, then 600 µl of chloroform was added, gently emulsified and then centrifuged at 10,000 rpm for 2 min. Aqueous phase containing DNA was transferred to 800 µl freshly prepared precipitation solution, mixed gently and centrifuged at 10,000 rpm for 2 min. DNA pellet was dissolved in 100 of 1.2 M NaCl by gentle vortexing. 300 µl of cold ethanol was added for 10 min for precipitation of DNA and was spin down at 10,000 rpm for 3–4 min. Ethanol was poured off and pellet was washed once with 70% cold ethanol and DNA was dissolved in 100 µl of sterile DNAs free deionized water and was stored at − 80 °C until it was used for amplification.

Conventional and semi-nested amplification of 18S ribosomal sub-unit (Ssu-rDNA) sequence

The amplification of DNA was performed as described earlier (Ijaz et al. 1998) by using different primers (Table 1). Ssu-rDNA amplification was carried out in 50 µl reaction mixture containing 5 µl of DNA template, 4 µl of buffer (50 mM KCl: 10 mM Tris-HCl: pH 8.3), 5 µl of MgCl₂ (1.5 mM), 5 µl of each dNTPs (200 µM), 1.6 µl of each primer (20 pmol) and 0.3 µl of Taq polymerase enzyme (0.5U) (Taq DNA polymerase, Fermentas Inc.) and 27.5 µl of deionized water. PCR reaction mixture tubes were transferred to a thermal cycler (Master Cycler, Eppendorf) and were subjected to 34 cycles of amplification. During each cycle, DNA sample was denatured at 94 °C for 60 s, annealed at 58 °C for 90 s and extension was done at 72 °C for 120 s. After completion of 34 cycles, 5 µl volume of each sample was electrophoresed (Electrophoresis System, C. B. S. Incl.) in 1.2% agarose gel and the products were visualized by staining the gel with ethidium bromide and photograph was taken (Gel Documentation System, Infinity Vilber Lourmat).

Semi-nested amplification was performed on the DNA template obtained from the conventional amplification. One µl of product from the first run was added to 49 µl of PCR mix at 82 °C (hot start method), containing the same ingredients and concentrations except for the primer pair (Table 1). The amplification conditions were identical except for 30 cycles. A negative control consisting of adding ultra pure water instead of template DNA to the PCR mixture was included in PCR amplification. After the completion of 30 cycles of amplification, semi-nested PCR product was analyzed by 1.2% agarose gel electrophoresis. Ethidium bromide stained gel was placed on UV trans-illuminator to observe the bands and a Polaroid photograph was taken by Gel Documentation System.

Restriction fragment length polymorphism (RFLP)

Nested products were digested with *Msp*I and *Eco*571 enzymes in buffer according to the manufacturer's specifications (Fermentas Inc. USA). In each case of digestion, a total of 20 µl reaction mixture was used. For digestion with *Msp*I, the reaction mixture contained 2 µl of Buffer Tango, 3 µl of nested PCR product, 2 µl of enzyme *Msp*I and 13 µl of deionized water. In case of digestion with *Eco*571, the reaction mixture contained 2 µl of Buffer G, 0.6 µl of SAM (S-adenosylmethionine, 0.01 mM), 3 µl of nested PCR product, 2 µl of enzyme *Eco*571 and 12.4 µl of deionized water. The reaction mixtures were left overnight in an incubator at 37 °C. Ten microlitres of each restricted sample was then mixed with 5 µl loading buffer and transferred onto a 2% agarose gel together with a 100 bp DNA ladder (Fermentas Inc. USA) for fragment size determination and a Polaroid photograph was taken by Gel Documentation System.

T. evansi specific amplification

As Ssu-rDNA amplification followed by RFLP analysis using *Msp*I and *Eco*571 restriction enzymes could only differentiate among *T. congolense*, *T. brucei* and *T. vivax* species. Therefore, for further characterization, Ssu-rDNA amplicon was subjected to PCR by using *T. evansi* specific TBR1 and TBR2 primers (Table 1) as reported previously by Ijaz et al. (6). The amplification conditions were same as described above.

Results

Microhematocrit centrifugation technique

Microscopic examination of Giemsa stained blood smears revealed 8% (24/300) animals having parasitaemia (Fig. 1)

Table 1 Primer sequences and predicted product size

Primers	Primer sequences	Product size
<i>For conventional amplification</i>		
18ST nF2 (F)	CAACGATGACACCCATGAATTGGGGA	750 bp
18ST nR3 (R)	TGCGCGACCAATAATTGCAATAC	
<i>For semi-nested amplification</i>		
18ST nF2 (F)	CAACGATGACACCCATGAATTGGGGA	650 bp
18ST nR2 (R)	GTGTCTTGTTCTCACTGACATTGTAGTG	
<i>For T. evansi DNA amplification</i>		
TBR 1 (F)	GAATATTAAACAATGCGCAG	164 bp
TBR 2 (R)	CCATTTATTAGCTTTGTTGC	

The primers were commercially prepared by Gene Link™

Fig. 1 Representative photomicrograph showing trypanosomes in Giemsa stained blood smear (magnification: 100x)

Fig. 2 Comparative prevalence of trypanosomiasis in different equine species as detected by MHCT, conventional and semi-nested PCR

and showed a variable prevalence among three species of equines (Fig. 2).

Conventional and semi-nested polymerase chain reaction (PCR)

Through these techniques, 21% (63/300) animals were found positive (Fig. 3a, b). These 21% positive cases also included the 8% positive samples diagnosed by the MHCT. The species wise detailed prevalence is shown in the Fig. 2.

Restriction fragment length polymorphism (RFLP)

RFLP analysis of the nested products by using restriction enzymes, *MspI* and *Eco57I*, resulted into nonspecific

Fig. 3 **a** Confirmation of trypanosomiasis by conventional polymerase chain reaction L1 = 100 bp DNA Ladder, L2 = positive control, L3 = negative control and L4, L5 = positive samples. **b** Confirmation of trypanosomiasis by semi-nested polymerase chain reaction L1 = 100 bp DNA ladder, L2 = positive control, L3 = negative control and L4, L5 = positive samples

restricted products which revealed that none of the species including *T. congolense*, *T. brucei*, *T. vivax*, *T. theileri*, and *T. vivax* were present in all 21% (63/300) positive animals having trypanosoma infestation.

T. evansi specific amplification

As *Ssu-rDNA* amplification followed by RFLP analysis using *MspI* and *Eco57I* restriction enzymes could only differentiate among *T. congolense*, *T. brucei*, *T. vivax*, *T. theileri*, and *T. vivax* species. Therefore, for further characterization, *Ssu-rDNA* amplicon was subjected to PCR by using *T. evansi* specific primers and it confirmed the presence of *T. evansi* infestation in all the positive animals (Fig. 4).

Fig. 4 Confirmation of *T. evansi* by using species specific primers M = 100 bp DNA ladder, 1 to 7 = positive samples, 8 = negative control, 9 = positive control

Discussion

Several studies in Pakistan reported that trypanosomiasis is endemic in horses (Murtaz et al. 2006). Previously, throughout the country, there has been no reported study on donkeys and mules being infested with this parasite. This is the first report about the prevalence of trypanosomiasis in donkeys and mules. Microscopy of Giemsa stained blood smears is easy and conventional technique for detecting hemoparasites in field conditions but its sensitivity is very low. MHCT method of buffy coat examination has improved the sensitivity of the microscopic methods as described by Woo (1969). In the current study, as compared to MHCT, PCR proved to be very sensitive and indicated a prevalence of 21% in the equines while MHCT could only detect trypanosomes in 8% of the samples. The higher prevalence rate detected by PCR was due to the higher sensitivity of the molecular technique, which can detect even less than one trypanosome per milliliter under experimental conditions (Singh et al. 2004). Prevalence of trypanosomiasis in horses has been reported earlier by Muieed et al. (2010), who reported a prevalence of 16%, which is in close compliance with current study which revealed a prevalence of 12% in the same species. In other studies conducted by Aslam et al. (2010) and Nadeem et al. (2011) reported 30.8% and 6.0% positive animals, however, the results of the current study are not in agreement with these studies. This variation may be due to differences of technique used and season of the study. In the present study, further analysis through RFLP and species specific PCR confirmed the *T. evansi* as the only prevalent species in the equines. This finding is also in compliance with previous study (Muieed et al. 2010).

We reported first time the prevalence of disease in donkeys and mules i.e. 3.67% and 5.33%, respectively, by using PCR. The study revealed comparatively low prevalence in donkeys as compared to horses and mules. Similarly, in a previous study, Faye et al. (2001) also reported that although donkeys are exposed to similar vector challenges as horses, they are radically less infected with trypanosomes.

Trypanosomiasis in donkeys and mules has been reported in other countries with variable prevalence. In a study in India, Ravindran et al. (2008) reported 6.8% prevalence in donkeys by using PCR while the blood smear examination method revealed 0.0% prevalence. In Ethiopia, Eyob et al. (2011) investigated the trypanosomal infection in equines including donkeys, horses and mules by phase contrast microscopy of buffy coat. They observed trypanosomes in 10.7% of the donkeys whereas horses and mules were found negative. The results differ from the current study because of geographic variation and differences in the techniques used. In another study in Gambia (Pinchbeck et al. 2008) horses and donkeys were screened using whole genome amplification (WGA) followed by trypanosome species identification using PCR. They reported an overall prevalence of trypanosomes as high as 91%, with an infection rate of 31% for *T. congolense* Savannah, 87% for *T. vivax* and 18% for *T. brucei*. Herrera et al. (2005) reported a 73% prevalence of *T. evansi* infection in horses and stated that low parasitaemia was only detected by PCR. In these studies, prevalence is very high as compared to the current study due to geographic variation, more vectors presence and differences in techniques. In previous studies involving PCR–RFLP, Desquesnes et al. (2001) reported a pan-trypanosome PCR test based on ITS-1 region of the ribosomal genes. They elaborated that the assay was three to five times cheaper than using the classical species specific primers, but it lacked sensitivity, especially for *T. vivax*. In another study, Geysen et al. (2003) successfully characterized different trypanosome species infecting cattle like *T. congolense*, *T. theileri*, *T. brucei* and *T. vivax* by using PCR–RFLP approach.

It is inferred from the current study that PCR–RFLP is a superior and far more sensitive assay as compared to Giemsa stained blood smear examination. The test can be used for early diagnosis of the trypanosomiasis and screening out the carrier animals (reservoirs of the infection). It is highly sensitive, universally applicable and has various possibilities for restriction enzyme analysis of the amplified region depending on the trypanosome species. Furthermore, it is concluded that *T. evansi* is the most prevalent species of trypanosoma in equines in Punjab and the strategies to control the infestation should be designed accordingly to improve the health and working efficiency of the equines. Further studies on the possibilities of use of other restriction enzymes may help to improve the species specificity of the PCR–RFLP assay.

Acknowledgements The authors highly acknowledge the generous funding by Higher Education Commission, Islamabad, Pakistan and also acknowledge the cooperation extended by the administrative and technical staff of the Outdoor Veterinary Hospital, UVAS, Society for the Prevention of Cruelty to Animals (SPCA), and Brooke's Hospital of Lahore for assistance in sample collection.

Authors' contribution NS conducted the experiments, ZIC supervised the experiments, AA and KM were co-supervisors, MS and IA helped in experiments, AH and SAK reviewed the manuscript before submission.

Compliance with ethical statement

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experimental design and protocol was submitted to the directorate of advanced studies and research, UVAS, Lahore and was duly approved before the start of the study.

Informed consent Informed consent was obtained from all of the owners of the animals included in the study.

References

- Anonymous (2016) Economic survey of Pakistan 2015–2016. Finance Division, Economic Advisors Wing, Ministry of Finance, Government of Pakistan, Islamabad Chapter 2, p 38
- Anwar M, Mohammed CG (1986) An outbreak of trypanosomiasis in donkeys. *Pak Vet J* 6:50
- Aslam A, Chaudhry ZI, Reman HU, Ashraf K, Ahmad N, Yaqub T, Maqbool A, Shakoori AR (2010) Comparative evaluation of parasitological, serological and DNA amplification methods for diagnosis of natural trypanosomal infection in equines. *Pak J Zool* 42:371–376
- Bano L, Jan AH (1986) Natural occurrence of trypanosomiasis in ungulates of N. W. F. P. (Pakistan). *Pak Vet J* 6:137–139
- Benjamin MM (1986) Outline of veterinary clinical pathology, 3rd edn. The Iowa State University Press, Ames, pp 05–37
- Biryomumaisho A, Eli-Katunguka R, Melville SE, Cailleau A, Lubega GW (2013) Livestock trypanosomosis in Uganda: parasite heterogeneity and anaemia status of naturally infected cattle, goats and pigs. *Parasitol Res* 4:1443–1450
- De Almeida PJ, Ndao M, Van Meirvenne N, Geerts S (1997) Diagnostic evaluation of PCR in goats experimentally infected with *Trypanosoma vivax*. *Acta Trop* 66:45–50
- Desquesnes M, Tresse L (1996) Evaluation of sensitivity of PCR for detecting DNA of *Trypanosoma vivax* with several methods of blood sample preparations. *Rev Elev Med Vet Pays Trop* 49:322–327
- Desquesnes M, McLaughlin G, Zoungana A, Dávila AMR (2001) Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. *Int J Parasitol* 31:610–614
- Eyob A, Mekuria S, Regassa A, Abebe R (2011) A cross-sectional study of equine trypanosomosis and its vectors in Wolayta zone, Southern Ethiopia. *J Vet Med Anim Health* 3(2):21–26
- Faye D, De Almeida PJ, Goossens B, Osaer S, Ndao M, Berkvens D, Speybroeck N, Nieberding F, Geerts S (2001) Prevalence and incidence of trypanosomosis in horses and donkeys in Gambia. *Vet Parasitol* 101:101–114
- Geysen D, Delespau V, Geerts S (2003) PCR–RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. *Vet Parasitol* 110:171–180
- Gill BS (1991) Trypanosomes and trypanosomosis of Indian Livestock. Indian Council of Agricultural Research, New Delhi, pp 1–192
- Herrera HM, Norek A, Freitas TPT, Rademaker V, Fernandes O, Jansen AM (2005) Domestic and wild mammals' infection by *T. evansi* in a pristine area of the Brazilian Pantanal region. *Parasitol Res* 96:121–126
- Ijaz MK, Nur-E-Kamal MS, Muhammad AI, Dar FK (1998) Comparative on the sensitivity of polymerase chain reaction and microscopic examination for the detection of *Trypanosoma evansi* in experimentally infected mice. *Comp Immunol Microbiol Infect Dis* 21:215–223
- McLaughlin GI, Ssenyonga SS, Nanteza E, Rubaire-Akiki Wafula O, Hansen RD, Vodkin MH, Novak RJ, Gordon VR, Montenegro-James S, James M, Aviles H, Armijos R, Santrich C, Weigle K, Saravia N, Wozniak E, Gaye O, Mdachi R, Shapiro SZ, Chang KP, Kakoma I (1996) PCR based detection and typing of parasites. In: Zcel MA, Alkan MZ (eds) *Parasitology for the 20th century*. CAB International, Wallingford, pp 261–287
- Muieed MA, Chaudhry ZI, Shakoori AR (2010) Comparative studies on the sensitivity of polymerase chain reaction and microscopic examination for the detection of *Trypanosoma evansi* in horses. *Turk J Vet Anim Sci* 34:507–512
- Murtaz UH, Muhammad G, Gutierrez C, Iqbal Z, Shakoor A, Jabbar A (2006) Prevalence of *Trypanosoma evansi* infection in equines and camels in the Punjab Region, Pakistan. *Ann N Y Acad Sci* 1081:322–324
- Nadeem A, Aslam A, Chaudhary ZI, Ashraf K, Saeed K, Ahmad N, Ahmed I, Rehman HU (2011) Prevalence of Surra in equines using indirect fluorescent antibody technique. *Pak Vet J* 31:169–170
- Nantulya VM (1990) Trypanosomiasis in domestic animals: the problems of diagnosis. *Rev Sci Tech* 9:357–367
- Njiru ZK, Constantine CC, Guya S, Crowther J, Kiragu JM, Thompson RCA, Dávila AMR (2005) The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. *Parasitol Res* 3:186–192
- Olaho-Mukani W, Munyua WK, Mutugi MW, Njogo AR (1993) Comparison of antibody and antigen detection enzyme immunoassay for the diagnosis of Trypanosomiasis in camels. *Vet Parasitol* 45:231–240
- Pinchbeck GL, Morrison LJ, Tait A, Langford J, Meehan L, Jallow S, Jallow J, Jallow A, Christley RM (2008) Trypanosomosis in The Gambia: prevalence in working horses and donkeys detected by whole genome amplification and PCR, and evidence for interactions between trypanosome species. *BMC Vet Res* 4:7
- Ravindran R, Rao JR, Mishra AK, Pathak KML, Babu N, Satheesh CC, Rahul S (2008) *Trypanosoma evansi* in camels, donkeys and dogs in India: comparison of PCR and light microscopy for detection. *Vet Arhiv* 78:89–94
- Singh N, Pathak KML, Kumar R (2004) A comparative evaluation of parasitological, serological and DNA amplification methods for diagnosis of natural *Trypanosoma evansi* infection in camels. *Vet Parasitol* 126:365–373
- Waheed MA, Khan IA, Khan MH (1998) A report on Surra in Gujranwala. *Pak Vet J* 18:170–172
- Woo PTK (1969) The hematocrit centrifuge for the detection of trypanosomes in blood. *Can J Zool* 47:921–923