
http://researchrepository.murdoch.edu.au/8678/

Copyright: © 2012 Elsevier B.V..

It is posted here for your personal use. No further distribution is permitted.

http://researchrepository.murdoch.edu.au/8678/

Copyright: © 2012 Elsevier B.V..

It is posted here for your personal use. No further distribution is permitted.
Microsatellite typing and population structuring of *Trypanosoma evansi* in Mindanao, Philippines

McInnes, L.M.¹*, Dargantes, A.P.¹², Ryan, U.M. ¹ and Reid, S.A.³

¹Division of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Perth WA 6150

²College of Veterinary Medicine, Central Mindanao University, University Town, Musuan, Bukidnon, Philippines 8710

³Public Health Division, Secretariat of the Pacific Community, B.P. D5, 98848 Noumea cedex, New Caledonia

* Author for correspondence:

Linda M. McInnes

Division of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Perth WA 6150

Phone: +61893602495

FAX: +61893104144

Email: LindaMMcInnes@gmail.com
ABSTRACT

Trypanosoma evansi, a blood-borne protozoan parasite with an extensive geographical range is the causative agent of the livestock disease known as surra. A total of 140 out of 179 T. evansi isolates collected between 2006 and 2007 from 44 villages (comprising of 16 reported surra outbreaks) in 3 provinces (Agusan del Sur (ADS), Surigao del Sur (SDS) and Agusan del Norte (ADN)) in Mindanao, Philippines were each successfully genotyped using a suite of 7 polymorphic microsatellites. The study identified 16 multi locus genotypes (MLG) within the T. evansi isolates and evidence of the spread of surra outbreaks from one village to another, most likely due to the movement of infected animals. Genotyping provided evidence of population sub-structuring with 3 populations (I, II and III (only 1 isolate)) identified. The most abundant population was II, which was the predominant population in ADS and SDS ($p=0.022$). In addition, buffalo mortality was statistically higher in outbreak areas associated with isolates from population I (13.6%) than with isolates from population II (6.9%) ($p=0.047$). The present study has highlighted the utility of microsatellite loci to improve understanding of the epidemiology of T. evansi and in tracking surra outbreaks.

KEYWORDS

Trypanosoma evansi, surra, epidemic, microsatellites, DNA, Mindanao, Philippines.
1. Introduction

*Trypanosoma evansi* is endemic in Africa, the Middle East, Central and South America, and many parts of Asia including the Philippines (OIE, 2008). *Trypanosoma evansi*, although able to infect most classes of mammals, is principally a livestock disease (surra), causing considerable mortality and morbidity in bovines, equines and camelids worldwide. In the Philippines, surra outbreaks have been reported in all 13 regions and resultant mortalities in horses, buffaloes and cattle between 1989 and 1997 have been estimated at a cost of US$1.15 million per annum (Manuel, 1998). There has recently been a large number of severe outbreaks in the Philippines with an outbreak in the North Samar province reported to have caused buffalo mortalities in excess of a thousand over a 6-month period (Cresencio et al., 1994; Manuel, 1998).

Surra was first introduced into the Philippines in the early 19th century with the importation of infected cavalry horses from China by the American forces during the American-Spanish war (Manuel, 1998). The initial and successive surra cases which occurred in the Philippines were predominately reported as high mortalities of equines (Manresa, 1935; Randall and Schwartz, 1936; Manuel, 1998). Mindanao, the second largest and southernmost island in the Philippine archipelago appeared to be free of surra until 1989 when the first reported cases of surra (38% equine and bovine mortality) were recorded in the southern Mindanao province of Sarangani (Mercado, R. pers. comm., 2010). Subsequent to the Sarangani outbreak, further surra outbreaks have occurred in a range of Mindanao provinces (Fig. 1). At present, the economic losses due to surra in Mindanao have been estimated at US$1.58 million per year from every 10 villages where the disease is moderately to highly prevalent (Dobson et al., 2009).

Surra presents as a highly variable clinical disease with the variation in disease manifestation (acute, sub-acute or chronic) associated with differences in host susceptibility.
and/or parasite strain virulence (OIE, 2010). An important feature of *T. evansi* infections is the sporadic nature of surra outbreaks which are often localised. High mortalities are most often attributed to the movement of infected animals into areas previously free from infection or the movement of surra-free animals into endemic areas (Payne et al., 1988). The endemic nature of *T. evansi* in the absence of high mortality outbreaks has led to speculation that surra may be enzootically stable in some areas such as Indonesia (Payne et al., 1991a; Payne et al., 1991b).

Microsatellites, which are highly discriminatory genetic tools have been used to examine *T. cruzi* phylogeography (Llewellyn et al., 2009a; Llewellyn et al., 2009b), differences in *T. cruzi* clinical disease (D'Avila et al., 2009), *T. brucei gambiens*e population structuring (Koffi et al., 2009; Simo et al., 2010), *T. brucei* mixed strain infection investigations (Balmer and Caccone, 2008), mating in *T. congolense* (Morrison et al., 2009) and *T. evansi* population structuring in Kenya (Njiru and Constantine, 2007; Salim et al., 2011). As *T. evansi* is widely believed to have evolved from *T. brucei* which was spread beyond the tsetse fly belt (Hoare, 1972). The genetic similarity between *T. brucei* and *T. evansi* has led others to propose that *T. evansi* should be considered a subspecies of *T. brucei* rather than a species (Lai et al., 2008). This genetic similarity between *T. brucei* and *T. evansi* means the *T. brucei* genome is highly useful for development of *T. evansi* genetic tools. In the present study, microsatellite markers developed from *T. brucei* genetic information were used to characterise the isolates and parasite populations from barangays (the Philippines’ equivalent of a village) without surra outbreaks and those isolates involved in the late 2005 to 2007 *T. evansi* outbreaks in 16 barangays in Mindanao, Philippines and their possible association with clinical disease, patent parasitaemia and temporal and spatial sub-structuring.
2. Materials and methods

2.1. Sources of isolates and sample collection

A total of 179 *T. evansi* isolates were collected from infected livestock (mostly buffaloes) from 44 barangays (with or without surra outbreaks) in the provinces of Agusan del Sur (ADS), Surigao del Sur (SDS) and Agusan del Norte (ADN), as shown in Figures 2a and b, in Mindanao, southern Philippines during surra epidemiological surveys in 2006–2007. The field surveys were conducted in a total of 89 barangays from 36 municipalities in 5 provinces in Mindanao involving more than 3,000 village livestock (Dargantes, 2010). From 16 surra outbreak barangays (Table 1), 660 local swamp buffaloes (*Bubalis bubalis*), 24 cattle, 50 horses and 33 goats were randomly bled. Whole blood (6-9 mL) was aseptically collected from the jugular vein of livestock into a labelled ethylenediamine-tetra acetic acid (EDTA) vacutainer vial (Vacuette®, Greiner Bio-One, USA). Blood samples were placed inside a cooler box until examination. The microhaematocrit centrifugation technique (MHCT) and mouse inoculation test (MIT) were used to detect *T. evansi* in blood samples as described in Dargantes et al. (2009). Mice parasitaemic with *T. evansi* were bled and the blood was stored in EDTA vials at -20°C until required.

Information on each animal sampled including geographical origin (barangay, municipality and province), MHCT parasite enumeration, card agglutination test for *Trypanosoma evansi* (CATT), host species, blood packed cell volume (PCV), age and body condition was recorded. Data on the percentage mortality of buffaloes, cattle, horses and goats in conjunction with the outbreaks of surra were obtained from the municipal veterinary personnel or local barangay officials. All field and laboratory activities were conducted with approval from the Murdoch University Animal and Human Ethics Committees (Permit numbers R881/01 and 2007/034, respectively).
2.2. **DNA extraction**

Whole genomic DNA was extracted from blood collected from mice inoculated with the blood of a *T. evansi*-infected original host (M) and from the whole blood from the corresponding original host (OH) using a modified protocol with a MasterPure™ DNA Purification Kit (EPICENTRE® Biotechnologies, Madison, Wisconsin, U.S.A.) as described by McInnes et al. (2011). DNA was stored at -20°C until use.

2.3. **Microsatellite typing tool development**

Microsatellites were identified from *T. brucei* genome sequences sourced from GenBank by screening for repeating DNA motifs utilising the software program Tandem Repeats Finder (Benson, 1999). Primers were designed in flanking regions of selected microsatellites chosen on the basis of perfect repeating motifs, originating from different contigs and where possible from different chromosomes to minimise the chance of linkage. An initial 15 microsatellites were chosen for analysis (11 dinucleotide microsatellites and 4 trinucleotide microsatellites) and primers were designed with the aid of software program Amplify v3.1. Primers were tested on 3 different *T. evansi* isolates (collected in different years from livestock in Central, and East Java and Sumbawa, Indonesia) previously shown to be genetically different (C. Constantine, pers. comm., 2007) and the resultant amplicons electrophoresed on 4% High resolution agarose (Fisher Biotec, Australia) to ascertain the best candidates for further analysis. Promising microsatellites, which appeared to display some degree of heterogeneity amongst the three isolates, were chosen for fluorescent labelling and multiplexing. Of the 15 microsatellites, only 4 were deemed useful after evaluation was completed on 20 *T. evansi* isolates (11 loci were discarded due to monomorphism, unscoreable results and poor PCR amplification). Chosen loci
Tbb1 and Tbb10 were combined in one multiplex (TEM1) and Tbb5 and Tbb9 in a second multiplex (TEM2) with fluorescent labels as indicated in Table 2.

All designed primers were tested on the DNA extracted from the blood of a mouse experimentally infected with a *T. evansi* isolate using the following PCR conditions: 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 56°C for 20 sec and 72°C for 30 sec, finishing with a final extension of 72°C for 45 min. All multiplex PCRs were carried out in 25 µL reactions comprising of 1 x PCR buffer, 1.5 mM MgCl₂, 0.1 mM of each dNTPs, 0.4 µM of each primer, 0.04 units of Tth+ DNA polymerase (Fisher Biotec, Australia) and 1 µL DNA template. All PCR amplicons generated from this *T. evansi* isolate were purified using a MO BIO UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories Inc. West Carlsbad, California, USA) and sequenced using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosoystem 3730 DNA Analyzer to confirm PCR amplification of correct loci.

2.4. Microsatellite typing of samples

DNA extracted from the Philippine *T. evansi* samples (both M and OH samples) were amplified using the two developed multiplexes (TEM1 and TEM2) following conditions described above as well as with microsatellite primers designed by Biteau et al. (2000) for the following loci: MORF-CA, MT3033-AC/TC, M6C8-CA, MT3033-TA and MEST19-AT/GT. The five loci identified by Biteau et al. (2000) were multiplexed to conserve resources. Loci MORF-CA and MT3033-AC/TC were combined in one multiplex (Bite1) with MORF-CA fluorescently labelled with 6-FAM (Applied Biosystems, Life Technologies, Carlsbad, California) and MT3033-AC/TC with Vic (Applied Biosystems). Loci M6C8-CA, MT3033-TA...
and MEST19-AT/GT were combined in a second multiplex (Bite2) with the forward primers for M6C8-CA labelled with NED (Applied Biosystems), MT3033-TA with Vic (Applied Biosystems) and MEST19-AT/GT with 6-FAM (Applied Biosystems). Conditions for the Bite1 and Bite2 multiplexes were as follows: 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 53°C for Bite1 and 50°C for Bite2 for 30 sec and 72°C for 30 sec, and a final extension of 72°C for 45 min. The PCR reaction reagents for both Bite1 and Bite2 were the same as previously described for the TEM1 and TEM2 multiplexes. All fluorescently labelled PCR amplicons were analysed on an ABI 3730 DNA Analyzer (Applied Biosystems Inc.) in conjunction with a GeneScan™ 600 LIZ® Size Standard (Applied Biosystems). Allele identification was performed manually using the ABI program Genemapper v3.5 (Applied Biosystems). The term multilocus genotype (MLG) is applied in this paper to isolates with identical genotype established from several genetic loci.

2.5. Population analysis

Population structuring analyses of datasets using the 4 (developed as part of the present study) and 7 loci (the 4 developed loci and 3 loci published by Biteau et al. (2000) were conducted using the software program Structure 2.3.3, which is a Bayesian model-based clustering method for inferring population structure and assign individuals to populations from multilocus genotype data (Pritchard et al., 2000). Simulations using the admixture and correlated frequencies model were run for K=1-7 with a burn in period of 20,000 and 100,000 MCMC repetitions and the average log probability of data clustering (L(K)) from 15 replicates calculated.
The construction of Neighbour-Joining (NJ) phylogenetic trees of microsatellite data from *T. evansi* isolates were carried out using Powermarker 3.25 (Liu and Muse, 2005), and the trees visualised using MEGA 4.1 (Tamura et al., 2007). The NJ genetic distance trees were constructed with the shared allele model (*D*<sub>AS</sub>) (Jin and Chakraborty, 1994) and 1,000 bootstrap replicates for all isolates typed at the 4 loci (TEM1 and TEM2) (159 isolates) and with all 7 loci (140 isolates).

Microsatellite allelic frequencies, Nei’s unbiased measures of genetic distance and inbreeding coefficient (*F*<sub>IS</sub>), Wright’s fixation index (*F*<sub>ST</sub>) values and departure from Hardy–Weinberg equilibrium (heterozygote deficiency or excess) were calculated with the genetic software GENEPOP v.3.4 (http://genepop.curtin.edu.au) originally developed by Raymond and Rousset (1995). In addition, the standardised index of association (*I*<sub>AS</sub>), a measure of the association between alleles at different loci, was calculated using the software program LIAN - LInkage ANalysis, Version 3.5 (http://adenine.biz.fh-weihenstephan.de/cgi-bin/lian/lian.cgi.pl) developed by Haubold and Hudson (2000). The null hypothesis of linkage equilibrium for the *T. evansi* dataset was tested by Monte Carlo simulation (1,000 iterations) and a parametric test (Haubold et al., 1998). The multi locus genotype (MLG) index (*G/N*), a measure of clonality expressed as a fraction derived from the number of MLGs (*G*) compared with the number of isolates (*N*), was calculated. Genic differentiation with calculation of *F*<sub>ST</sub> values between populations (determined by Structure 2.3.3), provinces, year of sampling and provinces together with years of sampling were calculated using GenAlEx (Peakall and Smouse, 2006).

2.6. *Statistical analysis*
The populations identified amongst the isolates were recorded and coded together with other information gathered about each host in a dataset using the Statistical Package for Social Sciences (SPSS) 17.0.1 (SPSS Inc., Chicago, IL, USA). The statistical significance of any differences in values for categorical variables (population, geographical origin, MHCT, CATT and body condition) was determined using Pearson’s Chi-square ($\chi^2$) ($p<0.05$) and for continuous variables (host animal age, mortality and PCV) using Student’s T-test ($p<0.05$).

Analysis of the predominant *T. evansi* population (as determined by Structure 2.3.3), and buffalo mortality required the removal of any sampling locations that could not be assigned a predominant population (i.e. barangays with 2 isolates, each assigned by Structure 2.3.3 to different populations). This reduced the dataset analysed from 44 to 41 barangays.

2.7. **Geographical representation of *T. evansi* microsatellite information**

The spatial relationships of the *T. evansi* isolate genotypes were visualised by plotting populations, epidemics and buffalo mortality figures together with isolate genotypes on a map of Mindanao using a geographical information system (GIS) program Quantum GIS 1.5.0-Tethys (http://qgis.org/).

3. **Results**

3.1. **Microsatellite selection procedure**

The loci M6C8 and MT3033TA sourced from Biteau et al. (2000) which were initially combined in two multiplexes, were monomorphic in 120 Mindanao *T. evansi* isolates and these loci were therefore discarded.
DNA sequencing of amplicons of each of the loci (Tbb1, Tbb5, Tbb9, Tbb10) developed in the present study confirmed that the loci amplified from *T. evansi* isolates were the same loci identified from the *T. brucei* genome sequence data (data not shown). Therefore a total of 7 loci (4 developed during the present study and 3 sourced from Biteau et al. (2000)) were used for further analysis.

3.2. Microsatellite typing of original host (OH) and mouse derived (M) DNA extracts

Microsatellite typing with the multiplexes TEM1, TEM2, Bite1 and Bite2 were attempted on 262 DNA samples from 195 isolates: 179 *T. evansi* mouse derived (M) isolates and 99 *T. evansi* original host derived (OH) samples. Of the 179 *T. evansi* M isolates, 83 (46%) had a corresponding OH sample, i.e. DNA extractions from the blood of an infected buffalo and the blood of a mouse infected with a sample from the same buffalo. *Trypanosoma evansi* M samples were more successfully amplified than the OH samples; 7 loci were amplified from 70.9% (127/179) M isolates but only 13.1% (13/99) OH isolates were amplified at all 7 loci.

Of the 195 isolates tested, 6 isolates typed had 3-4 alleles at each microsatellite locus typed. Two OH isolates (A7B-387OH and A73-807OH) and 4 M isolates (A7E-788M, A7E-859M, A7E-1079M and SN7-151M) demonstrated mixed loci. The complementary M isolates (A7B-387M and A73-807M) to the two mixed OH isolates (A7B-387OH and A73-807OH) did not demonstrate the same mixture of alleles but contained alleles present in the OH isolate mixtures.

3.3. Population structuring
An unrooted genetic distance tree with NJ 1000 bootstrap was constructed with the shared allele evolutionary model from 140 isolates typed at 7 loci (Fig. 3) and 179 isolates at 4 loci (not shown). Population structuring analyses conducted on 4 and 7 loci datasets using the software program Structure 2.3.3 (Pritchard et al., 2000) were congruent (i.e. T. evansi isolates which were present in both datasets, were assigned to the same populations in both analyses).

Furthermore, the highest average log probability of data clustering (L(K)) from 15 replicates calculated for the 7 loci dataset was determined for K=3 (-435.867). The three populations identified were designated I, II and III and are represented in the constructed NJ genetic distance tree (Fig. 3). The predominant population in the 7 loci dataset was population II which comprised 67.1% (94/140) of isolates followed by population I with 32.1% (45/140) of isolates and population III with 0.7% (1/140) of isolates. The analysis also identified 16 MLGs (sets of isolates that appeared to be identical when typed with a given set of genetic markers in a basically clonal species) (Tibayrenc and Ayala, 1991) within the three populations (I, II and III). The largest MLG groups were A and C; MLG A comprised 21 identical isolates and MLG C had 55 isolates.

3.4. Population analysis

Analysis of the 140 isolates typed at 7 loci revealed complete heterozygosity at all of the 7 microsatellite loci analysed (no homozygotes present in any loci in any isolate genotyped) with significant deviations from Hardy-Weinberg equilibrium (p<0.001). The mean inbreeding coefficient (FIS) of the 7 loci was -0.3458 and negative FIS values were also generated for each individual locus. Levels of polymorphism varied between loci, with the total number of alleles per locus ranging from 3 (MORFCA) to 12 (Tbb5). The MLG index (G/N) for 7 loci was 0.14 (6
unique and 14 repeated MLGs). The standardised index of association ($I_A^S$) for the complete dataset was 0.6956 with significant linkage disequilibrium amongst all combinations of loci ($p<0.001$).

The expected and observed heterozygosity, number of alleles and $F_{IS}$ values of each locus within populations I, II and III are presented in Table 3. Nei’s genetic distance, $F_{ST}$ and geneflow ($N_M$) of isolates divided by population (I and II; III excluded due being only 1 isolate), province (ADS and SDS), year of collection (2006 and 2007), or combination of province and year of collection (ADS-2006, ADS-2007, SDS-2006 and SDS-2007) were calculated (Table 4). There was little evidence in population structuring according to province or year of collection (0.022 and 0.021 respectively). There were significantly different $F_{ST}$ values (0.118-0.154) generated for isolates collected from ADS in 2006 compared with those collected from ADS in 2007 or from SDS in either 2006 or 2007. The $F_{ST}$ value between populations I and II was 0.117. There was a significant linkage of all loci for isolates divided on the basis of Structure 2.3.3 population, province, year of collection or province and year of collection based on calculated $I_A^S$ values ($p<0.001$).

3.5. Statistical and geographical analysis

No significant differences were seen between population I and II isolates (III was omitted due to insufficient data) and province in regard to any categories except for province and buffalo mortalities ($p<0.05$). Population II was detected in a significantly higher proportion of samples from ADS (58.7%, 95% C.I. 48.9-68.1%) and SDS (81.3% 95% C.I. 67.4-91.1%) ($p=0.022$) (Fig. 4). The mean buffalo mortality in locations with a predominant population I of $T. evansi$
isolates was significantly higher (13.6%) compared to locations with a predominant II population of *T. evansi* isolates (6.9%) (*p*=0.047).

Analysis of MHCT, CATT, buffalo mortality and PCV with *T. evansi* MLGs could only be conducted for two MLGs; A and C (sufficient numbers for analysis), which belonged to the same population (II). MLG A which comprised 21 isolates was primarily isolated in Surigao del Sur, whilst MLG C which comprised 55 isolates was predominantly isolated in Agusan del Sur (Fig. 6). There were no significant differences on the MHCT and CATT results and PCV values of hosts infected with either MLG A or C. Buffalo mortality in areas with a reported outbreak had significantly higher mortality when MLG A *T. evansi* isolates were present than in areas where MLG C isolates were present (*p*<0.05). The mean buffalo mortality involving MLG A isolates was 17.1% and 5.9% for MLG C isolates. There was a more visible association between geographical location of MLG A isolates and areas of recorded buffalo mortality (4 barangays) compared with MLG C and areas of buffalo mortality (1 barangay) (Fig. 5).

### 3.6. Correlation between epidemic and *T. evansi* MLG

Samples were collected from livestock within 3 months of first outbreak report in 9 of the 17 reported outbreaks of surra. The *T. evansi* MLG most likely to be the source of the outbreak could not always be inferred because many of the epidemic sites sampled yielded too few isolates to be conclusive. Several isolates of the same MLG were typed in different outbreaks and isolates collected from some outbreaks comprised more than one *T. evansi* genotype as presented in Table 5.

### 4. Discussion
The population structure of *T. evansi* in Mindanao exhibited evidence of clonal expansion, in particular within epidemics with the observation of 14 MLGs in the dataset as well as significant linkage, negative $F_{IS}$ values and 100% heterozygosity at each locus. High heterozygosity occurs in clonal organisms due to accumulations of random mutation events (Halkett et al., 2005; De Meeus et al., 2006). Due to the absence of the tsetse fly, vector in which *T. brucei* is known to undergo sexual recombination (Peacock et al., 2011), *T. evansi* within the Philippines and other non tsetse fly areas is inferred to be asexually reproducing. The complete heterozygosity observed in the *T. evansi* isolates from the Philippines is incompatible with meiotic segregation (Tibayrenc et al., 1990) and supportive of *T. evansi* reproduction being asexual in the Philippines. However, heterozygosity may also be due to the typing of mixed strain infections. The presence of 14 *T. evansi* MLGs in Mindanao isolates, however, negates the notion that the heterozygous microsatellite profiles produced are the result of mixed infections (the combination of two homozygote strains giving rise to two alleles at each locus). The finding of several MLGs amongst the Philippine isolates is in contrast to a recent study conducted by Salim et al. (2011) on *T. evansi* in camels within Sudan. Salim et al. (2011) noted that the absence of any MLGs in their dataset was unusual for what is believed to be a clonal organism. They argued that their dataset may have been subject to “frequent allelic dropouts, Wahlund effects or both”.

Mixed strain infections of *T. evansi* were evident in 6 isolates (the presence of 4 alleles at each locus), 2 of which originated from OH isolates. Mixed infections have been reported in cattle (*T. congolense*) (Morrison et al., 2009), humans (*T. brucei*) (Truc et al., 2002) and in pigs (*T. brucei*) (Jamonneau et al., 2003). Intra-host variation has been observed in the ribosomal internal transcribed spacer 1 of *T. evansi* from buffalo and cattle from Thailand, which could be
due to natural mixed strain infections (Khuchareontaworn et al., 2007; Areekit et al., 2008). Mixed trypanosome species and strain infections are also known to occur in vectors such as the tsetse fly (MacLeod et al., 1999). Tabanids are considered opportunistic feeders (Magnarelli and Anderson, 1980; Gouteux et al., 1989; Muzari et al., 2010) and recent studies have shown that mixed host blood meals are frequent in some tabanid species (Muzari et al., 2010).

It is important to recognise that in vivo systems such as the MIT may exert ‘selective pressures’ (Jamonneau et al., 2003) that may lead to a reduced recognition of naturally occurring mixed infections. Concentration of trypanosomes by methods such as MIT has however, been very important for trypanosome analysis due to the characteristic low parasitaemia that is observed in most naturally T. evansi-infected hosts. Direct amplification of microsatellites without the aid of any concentration methodologies or PCR sensitivity enhancing strategies is frequently difficult. Duffy et al. (2009) were only able to amplify a suite of 8 microsatellites from 31 of 304 (10%) T. vivax-positive DNA samples. Nested PCRs may have increased sensitivity without the need for a potentially selective concentration process such as MIT, however, the reported increases in sensitivity achieved by nested microsatellite PCR design vary. Valadares et al. (2008) reported a 100-1,000-fold increase in the sensitivity with typing T. cruzi from infected tissue samples, whilst Morrison et al. (2007) only reported a 5-fold increase in sensitivity with a nested PCR using dried blood on FTA filter cards. PCR detection on FTA cards can however be problematic as reported by Cox et al., (Cox et al., 2010) who observed that single punch nested PCR analyses for trypanosomes can lead to considerable underestimations of prevalence.

The three populations (I, II and III) identified amongst the T. evansi isolates genotyped from Mindanao may be evidence of the existence of only three T. evansi lineages in the
northeastern Mindanao provinces (ADS and SDS). The population analysis indicates that the
outbreaks investigated in ADS and SDS were primarily caused by two *T. evansi* lineages which
have differentiated over time into two populations (I and II). The isolate AS-76 from Mapaga,
Prosperidad, ADS was the only representative of *T. evansi* population III in Mindanao.
Identification of only one isolate for this population suggests that this isolate may be a recent
introduction to the northeastern Mindanao provinces (ADS and SDS). The genetic similarity of
AS-76 with an isolate LZ-1 (typed at 4 loci) from the Philippine island Luzon suggests that AS-
76 isolate may have originated from Luzon. Mindanao is, however, an island geographically
removed from Luzon with restrictions of animal movement due to Mindanao being considered
foot and mouth disease (FMD) free without vaccination by the Office Internationale des
Epizooties (OIE) since 2001 (OIE, 2001). It is therefore more likely that AS-76 is an isolate from
a lineage of *T. evansi* present in some of the Mindanao provinces not examined in this study. A
comprehensive survey to genotype *T. evansi* isolates from all Mindanao provinces as well as
other Philippine provinces would be required to elucidate the origins of the 3 Mindanao *T. evansi*
populations. The present study did not test any isolates from the southern Mindanao provinces
which were the first to report surra outbreaks.

The provinces of ADS and SDS in Mindanao, although situated adjacent to one another,
are separated by a mountain range with few connecting roads. The mountain range, lack of
interconnecting roads and the political insurgency in the region may have restricted animal
movement between the provinces and thereby reduced the spread of *T. evansi* strains between
provinces. However, comparison of isolates from these two provinces did not support the
hypothesis that there is a significant genetic divide in isolates (low $F_{ST}$ values of 0.022) with
considerable gene flow (11.3) measured between the provinces. This was also evident with *T.*
evansi population II isolates present in both provinces and significantly more prominent in both ADS and SDS provinces than population I. The transmission of T. evansi MLGs between provinces was exemplified by MLGs A and C with isolates of these MLGs present in both provinces on either side of the mountain range.

Significant variations on the $F_{ST}$ values of Mindanao T. evansi based on population substructuring analysis may suggest that the isolates collected from ADS in 2006 were different from the isolates collected from the same province in the following year as well as from isolates collected from SDS in either 2006 or 2007. However, this could be an anomaly attributed to the small sample size ($n=9$) of isolates collected from ADS in 2006.

The susceptibility to T. evansi infection varies depending on the host species. However, it is not clear whether host-specific strains exist in the areas sampled. It is interesting to note that the outbreak recorded in Ebro, San Francisco identified a T. evansi MLG (K) from 4 horses which was not present in any of the other surra outbreaks in nearby barangays during the same year. This may have been due to a strain more pathogenic to horses than to buffalo or simply due to successful containment of the Ebro outbreak. In the present study there were no villages sampled which contained both horse and buffalo surra strains. This was usually the result of high surra mortality in horses leading to the elimination of most horses from surra epidemic areas prior to sampling. The high susceptibility or possible high pathogenicity of T. evansi to horses was evident in the outbreaks in Ladgadan, Del Rosario, Mapaga and Causwagan, ADS which recorded equine mortalities in excess of 30%. In Banahao, Lianga, SDS, T. evansi isolates were however recovered from 2 horses which were identified as MLG C, the most likely causative agent of the large outbreak in buffalo in Guadalupe and Concordia. As the Guadalupe/Concordia
outbreak occurred immediately before the Banahao outbreak, it may be due to translocation of *T. evansi*-infected animals.

The evidence that some MLGs were localised to specific barangays is probably a reflection of the transmission dynamics of *T. evansi* that require close proximity between hosts (Barros and Foil, 2007). In Mindanao barangays, the mechanical transmission of *T. evansi* by tabanids is likely to be enhanced by the common practice of communal tethering of livestock in pastures often adjacent to or in close proximity to irrigation canals, swamps and rivers or by using a common wallowing pool as in the case of buffaloes (Dargantes, A. pers. comm., 2010).

Areas with abundant water are likely to have high population of tabanids, as most tabanids require water for breeding and development (Mitzmain, 1913; Service, 1986; McElligott and Lewis, 1996; Butt et al., 2008).

Transmission of *T. evansi* by animal translocation is a logical explanation for the outbreaks reported in Guadalupe and Concordia, Esperanza, ADS. These outbreaks occurred in villages geographically close to one another, around the same time of the year (Jan-Mar 2007) and were both strongly associated with *T. evansi* MLG C. The MLG finding and temporal and spatial proximity of the outbreaks suggest this was in fact a single connected event rather than two separate outbreaks. The analysis revealed that the MLG responsible for these two outbreaks was the same, which then caused an outbreak in Ladgadan, San Francisco, ADS in the following year. This may be due to the movement of an infected host from Guadalupe or Concordia to Ladgadan and seasonal fluctuations in tabanid abundance culminating in an outbreak the following year in a new location.

In the present study, increased buffalo mortality was more associated with *T. evansi* population I isolates than with population II isolates, and with MLG C isolates than with MLG A
isolates from population II. However, it is important to note that the variation in pathogenicity could be due to the many other potentially influencing factors such as immune status and other contributing health issues of the hosts. Microsatellite typing is able to assist in elucidating this issue but influencing factors would need to be controlled or a more extensive survey over a considerably longer period of time would be required. Data from the application of microsatellite typing of *T. evansi* isolates from Mindanao shows that outbreaks were caused by the same *T. evansi* strain (MLG C), and that the *T. evansi* strains in the northeastern Mindanao provinces represent three lineages. Furthermore, the present study has demonstrated the value of microsatellites for the study of complex infectious agents such as *T. evansi*. However, larger and more representative datasets are required to provide a more accurate description of the origin of outbreaks, the transmission factors promoting spread of outbreaks and the importance of strain variation in pathogenicity.

Acknowledgements
Field surveys and collection of isolates were carried out in coordination with the Mindanao Unified Surra Control Approach (MUSCA) group headed by Dr. R. Mercado and the help from veterinarians and staff of collaborating government agencies in Agusan del Norte, Agusan del Sur and Surigao del Sur, and the members of the CMU Surra Team. The authors are also grateful for the significant assistance of Drs. J.R. Dargantes, P. Calo, M. Samar, J.A. Abella, R. Sumagang, E. Igsoc, M. Pagobo and of D. Miguel and K. Quero. The financial support from the Australian Centre for International Agricultural Research (ACIAR), Central Mindanao University (CMU), International Foundation of Science (IFS), Murdoch University and the
Australian Biosecurity Cooperative Research Centre (AB-CRC) which contributed to the research presented in the article is acknowledged.

**Conflict of interest statement**

The authors of this article declare that they have no conflicts of interest with regard to the work presented herein.

**References**


Dargantes, A.P., 2010. Epidemiology, control and potential insect vectors of Trypanosoma evansi (surra) in village livestock in southern Philippines. Murdoch University, Perth.


McInnes, L., Hanger, J., Simmons, G., Reid, S., Ryan, U., 2011. Novel trypanosome Trypanosoma gilletti sp. (Sarcomastigophora: Trypanosomatidae) and the extension of the host range of Trypanosoma copemani to include the koala (Phascolarctos cinereus). Parasitology 138, 59-70.


Valadares, H.M., Pimenta, J.R., de Freitas, J.M., Duffy, T., Bartholomeu, D.C., Oliveira Rde, P.,
Pena, S.D., Macedo, A.M., 2008. Genetic profiling of *Trypanosoma cruzi* directly in
infected tissues using nested PCR of polymorphic microsatellites. Int. J. Parasitol. 38,
839-850.
Table 1

Details of the locations of putative *Trypanosoma evansi* outbreaks in 16 barangays in Mindanao, Philippines between late 2005 and 2007 and the associated livestock mortality percentages.

<table>
<thead>
<tr>
<th>Barangay</th>
<th>Municipality</th>
<th>Province</th>
<th>Epidemic Year</th>
<th>Epidemic Months</th>
<th>Mortality %</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Buffalo</td>
<td>Cattle</td>
<td>Goat</td>
<td>Horse</td>
<td></td>
</tr>
<tr>
<td>Parang</td>
<td>Cantilan</td>
<td>SDS</td>
<td>2005</td>
<td>Dec</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>San Isidro</td>
<td>San Francisco</td>
<td>ADS</td>
<td>2006</td>
<td>Jan-Mar</td>
<td>17.7</td>
<td>12.8</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Ebro</td>
<td>San Francisco</td>
<td>ADS</td>
<td>2006</td>
<td>Jan-Mar</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Libas Gua</td>
<td>San Miguel</td>
<td>SDS</td>
<td>2006</td>
<td>Jan-Mar</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Ladgadan</td>
<td>San Francisco</td>
<td>ADS</td>
<td>2006</td>
<td>Jul-Sep</td>
<td>5.9</td>
<td>5.4</td>
<td>0.0</td>
<td>40.0</td>
<td></td>
</tr>
<tr>
<td>Mamis</td>
<td>Barobo</td>
<td>SDS</td>
<td>2006</td>
<td>Jun-Aug</td>
<td>17.1</td>
<td>6.0</td>
<td>9.1</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Del Rosario</td>
<td>Sibagat</td>
<td>ADS</td>
<td>2006</td>
<td>Mar-Apr</td>
<td>14.6</td>
<td>0.0</td>
<td>0.0</td>
<td>35.0</td>
<td></td>
</tr>
<tr>
<td>Mapaga</td>
<td>Prosperidad</td>
<td>ADS</td>
<td>2006</td>
<td>Mar-Apr</td>
<td>8.3</td>
<td>0.0</td>
<td>0.0</td>
<td>35.7</td>
<td></td>
</tr>
<tr>
<td>Bayan</td>
<td>Marihatag</td>
<td>SDS</td>
<td>2006</td>
<td>Oct-Nov</td>
<td>7.9</td>
<td>0.0</td>
<td>100.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>San Ignacio</td>
<td>Trento</td>
<td>ADS</td>
<td>2007</td>
<td>Apr-Jun</td>
<td>16.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Dimasalang</td>
<td>San Luis</td>
<td>ADS</td>
<td>2007</td>
<td>Dec-Mar</td>
<td>6.0</td>
<td>17.9</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Concordia</td>
<td>Esperanza</td>
<td>ADS</td>
<td>2007</td>
<td>Jan-Mar</td>
<td>5.6</td>
<td>8.3</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Guadalupe</td>
<td>Esperanza</td>
<td>ADS</td>
<td>2007</td>
<td>Jan-Mar</td>
<td>6.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Mambalili</td>
<td>Bunawan</td>
<td>ADS</td>
<td>2007</td>
<td>Jan-Mar</td>
<td>5.1</td>
<td>4.9</td>
<td>0.0</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td>Banahao</td>
<td>Lianga</td>
<td>SDS</td>
<td>2007</td>
<td>Mar-May</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Causwagan</td>
<td>Talacogon</td>
<td>ADS</td>
<td>2007</td>
<td>Nov-Feb</td>
<td>14.0</td>
<td>0.0</td>
<td>0.0</td>
<td>41.7</td>
<td></td>
</tr>
</tbody>
</table>

NR: Not recorded
SDS: Surigao del Sur
ADS: Agusan del Sur
Table 2

Primer sequences and modifications, amplicon sizes, and repeat motifs of microsatellite multiplexes (TEM1 and TEM2) designed on the basis of *T. brucei* GenBank accession data.

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Primer name - sequence 5´-3´ (modifications: PIG tail, fluorescent label)</th>
<th><em>T. brucei</em> Amplicon size bp</th>
<th><em>T. brucei</em> Acc. no.</th>
<th><em>T. brucei</em> motif</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TEM1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tbb1</td>
<td>Tbb1F - TTTGAAGAATTGTACCTCGGTG'3 Tbb1R- gttctTTTCAACCGCTTATCAGC’3</td>
<td>192</td>
<td>AL92960</td>
<td>GT₉⁹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tbb10</td>
<td>Tbb10F- TGTGGCTCTACTTTCGTTG’3 Tbb10R- gttctTCCCCCTTGTACGTGGC’3</td>
<td>262</td>
<td>NW001076899</td>
<td>CA₄₈</td>
</tr>
<tr>
<td><strong>TEM2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tbb5</td>
<td>Tbb5F- CGCAACAACCTTTCACATAGC’3 Tbb5R- gttctTGAGGGTTCTCCCTACAC’3</td>
<td>240</td>
<td>AC159443</td>
<td>CA₅₀</td>
</tr>
<tr>
<td>Tbb9</td>
<td>Tbb9F- CTTTCGCCTGCTTTCGTTG’3 Tbb9R- gttctGTAGGTGCGATTAGGTTG’3</td>
<td>239</td>
<td>NW001076895</td>
<td>TG₅₁</td>
</tr>
</tbody>
</table>
Table 3

Characterisation of the 7 microsatellite loci used in the population analysis of 3 populations (I, II and III as determined by Structure 2.3.3 analysis) of *T. evansi* isolates from Mindanao, the Philippines.

<table>
<thead>
<tr>
<th>Population</th>
<th>Locus</th>
<th>Number of isolates</th>
<th>Number of Alleles</th>
<th>$H_o$</th>
<th>$H_e$</th>
<th>$F_{IS}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Tbb1</td>
<td>45</td>
<td>3</td>
<td>1.000</td>
<td>0.511</td>
<td>-0.957</td>
</tr>
<tr>
<td></td>
<td>Tbb10</td>
<td>45</td>
<td>3</td>
<td>1.000</td>
<td>0.511</td>
<td>-0.957</td>
</tr>
<tr>
<td></td>
<td>Tbb5</td>
<td>45</td>
<td>5</td>
<td>1.000</td>
<td>0.579</td>
<td>-0.726</td>
</tr>
<tr>
<td></td>
<td>Tbb9</td>
<td>45</td>
<td>3</td>
<td>1.000</td>
<td>0.540</td>
<td>-0.850</td>
</tr>
<tr>
<td></td>
<td>MORFCA</td>
<td>45</td>
<td>2</td>
<td>1.000</td>
<td>0.500</td>
<td>-1.000</td>
</tr>
<tr>
<td></td>
<td>MT3033</td>
<td>45</td>
<td>3</td>
<td>1.000</td>
<td>0.566</td>
<td>-0.768</td>
</tr>
<tr>
<td></td>
<td>MEST19</td>
<td>45</td>
<td>3</td>
<td>1.000</td>
<td>0.511</td>
<td>-0.957</td>
</tr>
<tr>
<td>II</td>
<td>Tbb1</td>
<td>94</td>
<td>8</td>
<td>1.000</td>
<td>0.789</td>
<td>-0.268</td>
</tr>
<tr>
<td></td>
<td>Tbb10</td>
<td>94</td>
<td>7</td>
<td>1.000</td>
<td>0.790</td>
<td>-0.267</td>
</tr>
<tr>
<td></td>
<td>Tbb5</td>
<td>94</td>
<td>11</td>
<td>1.000</td>
<td>0.871</td>
<td>-0.149</td>
</tr>
<tr>
<td></td>
<td>Tbb9</td>
<td>94</td>
<td>4</td>
<td>1.000</td>
<td>0.750</td>
<td>-0.334</td>
</tr>
<tr>
<td></td>
<td>MORFCA</td>
<td>94</td>
<td>3</td>
<td>1.000</td>
<td>0.619</td>
<td>-0.615</td>
</tr>
<tr>
<td></td>
<td>MT3033</td>
<td>94</td>
<td>9</td>
<td>1.000</td>
<td>0.826</td>
<td>-0.210</td>
</tr>
<tr>
<td></td>
<td>MEST19</td>
<td>94</td>
<td>8</td>
<td>1.000</td>
<td>0.788</td>
<td>-0.268</td>
</tr>
<tr>
<td>III</td>
<td>Tbb1</td>
<td>1</td>
<td>2</td>
<td>1.000</td>
<td>0.500</td>
<td>-1.000</td>
</tr>
<tr>
<td></td>
<td>Tbb10</td>
<td>1</td>
<td>2</td>
<td>1.000</td>
<td>0.500</td>
<td>-1.000</td>
</tr>
<tr>
<td></td>
<td>Tbb5</td>
<td>1</td>
<td>2</td>
<td>1.000</td>
<td>0.500</td>
<td>-1.000</td>
</tr>
<tr>
<td></td>
<td>Tbb9</td>
<td>1</td>
<td>2</td>
<td>1.000</td>
<td>0.500</td>
<td>-1.000</td>
</tr>
<tr>
<td></td>
<td>MORFCA</td>
<td>1</td>
<td>2</td>
<td>1.000</td>
<td>0.500</td>
<td>-1.000</td>
</tr>
<tr>
<td></td>
<td>MT3033</td>
<td>1</td>
<td>2</td>
<td>1.000</td>
<td>0.500</td>
<td>-1.000</td>
</tr>
<tr>
<td></td>
<td>MEST19</td>
<td>1</td>
<td>2</td>
<td>1.000</td>
<td>0.500</td>
<td>-1.000</td>
</tr>
</tbody>
</table>

$H_o$: observed heterozygosity, $H_e$: expected heterozygosity, $F_{IS}$: inbreeding coefficient.
Table 4

Nei’s genetic distance, Wright’s Fixation Index ($F_{ST}$) and gene flow ($N_m$) for the different divisions in *T. evansi* isolates collected in Mindanao, the Philippines and typed with 7 microsatellite loci. Isolates were grouped by province of origin (Agusan del Sur (ADS) or Surigao del Sur (SDS)) and/or collection year (2006 or 2007) or population structure as determined by software program Structure 2.3.3 (populations I and II).

<table>
<thead>
<tr>
<th>Isolates’ population divisions</th>
<th>Nei’s genetic distance</th>
<th>$F_{ST}$</th>
<th>$N_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADS / SDS</td>
<td>0.122</td>
<td>0.022</td>
<td>11.276</td>
</tr>
<tr>
<td>2006 / 2007</td>
<td>0.142</td>
<td>0.021</td>
<td>11.48</td>
</tr>
<tr>
<td>ADS-2006 / ADS-2007</td>
<td>0.893</td>
<td>0.118</td>
<td>1.87</td>
</tr>
<tr>
<td>SDS-2006 / SDS-2007</td>
<td>0.049</td>
<td>0.011</td>
<td>22.9</td>
</tr>
<tr>
<td>ADS-2006 / SDS-2006</td>
<td>1.204</td>
<td>0.154</td>
<td>1.37</td>
</tr>
<tr>
<td>ADS-2007 / SDS-2007</td>
<td>0.066</td>
<td>0.012</td>
<td>20.54</td>
</tr>
<tr>
<td>ADS-2006 / SDS-2007</td>
<td>0.903</td>
<td>0.124</td>
<td>1.77</td>
</tr>
<tr>
<td>ADS-2007 / SDS-2006</td>
<td>0.171</td>
<td>0.032</td>
<td>7.68</td>
</tr>
<tr>
<td>I / II (Structure populations)</td>
<td>0.259</td>
<td>0.067</td>
<td>3.476</td>
</tr>
</tbody>
</table>
Table 5

The 7 microsatellite multi locus genotypes (MLGs) and number of isolates from putative *Trypanosoma evansi* outbreaks occurring in 16 barangays in Mindanao, Philippines between late 2005 and 2007.

<table>
<thead>
<tr>
<th>Barangay</th>
<th>Municipality</th>
<th>Province</th>
<th>Epidemic Year</th>
<th>Months</th>
<th>MLG typed at 7 loci and number of isolates (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parang</td>
<td>Cantilan</td>
<td>SDS</td>
<td>2005</td>
<td>Dec</td>
<td>MLG I-2</td>
</tr>
<tr>
<td>San Isidro</td>
<td>San Francisco</td>
<td>ADS</td>
<td>2006</td>
<td>Jan-Mar</td>
<td>NT</td>
</tr>
<tr>
<td>Ebro</td>
<td>San Francisco</td>
<td>ADS</td>
<td>2006</td>
<td>Jan-Mar</td>
<td>MLG K-4</td>
</tr>
<tr>
<td>Libas Gua</td>
<td>San Miguel</td>
<td>ADS</td>
<td>2006</td>
<td>Jan-Mar</td>
<td>MLG I-1 &amp; MLG M-1</td>
</tr>
<tr>
<td>Ladgadan</td>
<td>San Francisco</td>
<td>ADS</td>
<td>2006</td>
<td>Jul-Sep</td>
<td>MLG E-1</td>
</tr>
<tr>
<td>Mamis</td>
<td>Barobo</td>
<td>SDS</td>
<td>2006</td>
<td>Jun-Aug</td>
<td>MLGA-10, MLG M-4 &amp; unique-2</td>
</tr>
<tr>
<td>Del Rosario</td>
<td>Sibagat</td>
<td>ADS</td>
<td>2006</td>
<td>Mar-Apr</td>
<td>NT</td>
</tr>
<tr>
<td>Mapaga</td>
<td>Prosperidad</td>
<td>ADS</td>
<td>2006</td>
<td>Mar-Apr</td>
<td>MLG J-1 &amp; unique-1</td>
</tr>
<tr>
<td>Bayan</td>
<td>Marihatag</td>
<td>SDS</td>
<td>2006</td>
<td>Oct-Nov</td>
<td>MLG E-2</td>
</tr>
<tr>
<td>San Ignacio</td>
<td>Trento</td>
<td>ADS</td>
<td>2007</td>
<td>Apr-Jun</td>
<td>MLG G-6, MLG H-5, MLG M-3 &amp; unique-1</td>
</tr>
<tr>
<td>Dimasalang</td>
<td>San Luis</td>
<td>ADS</td>
<td>2007</td>
<td>Dec-Mar</td>
<td>MLG C-3</td>
</tr>
<tr>
<td>Concordia</td>
<td>Esperanza</td>
<td>ADS</td>
<td>2007</td>
<td>Jan-Mar</td>
<td>MLG C-12, MLG L-1 &amp; unique-2</td>
</tr>
<tr>
<td>Guadalupe</td>
<td>Esperanza</td>
<td>ADS</td>
<td>2007</td>
<td>Jan-Mar</td>
<td>MLG C-20 &amp; MLG L-1</td>
</tr>
<tr>
<td>Mambalili</td>
<td>Bunawan</td>
<td>ADS</td>
<td>2007</td>
<td>Jan-Mar</td>
<td>NT</td>
</tr>
<tr>
<td>Banahao</td>
<td>Lianga</td>
<td>SDS</td>
<td>2007</td>
<td>Mar-May</td>
<td>MLG C-2</td>
</tr>
<tr>
<td>Causwagan</td>
<td>Talacogon</td>
<td>ADS</td>
<td>2007</td>
<td>Nov-Feb</td>
<td>MLG E-2 &amp; MLG L-1</td>
</tr>
</tbody>
</table>

NT: Not typed at all 7 loci

MLG: multi locus genotype

Unique: an isolate with a unique multi locus genotype
**Fig. 1.** A map of Mindanao in the Philippines showing the provinces where surra outbreaks have been reported in 1989-2009 (Dargantes, J.R., Mercado, R. and Alforque, J. pers. comm., 2010).
Fig. 2 a and b. The locations of *Trypanosoma evansi* isolate sampling and surra epidemics (late 2005-2007) in Mindanao, Philippines. a. Depicts the Philippines with an inlay box over an area of Mindanao which is enlarged in b. and contains marked *T. evansi* sampling locations (⊙) and epidemic locations (†).
**Fig. 3.** Neighbour-joining tree inferred from the Share allele ($D_{AS}$) genetic distances calculated for the microsatellite data of 140 *Trypanosoma evansi* isolates from Mindanao, Philippines genotyped at 7 loci. Unique isolates are identified by the individual code (i.e. A7E-912M) and MLGs (groups of identical isolates) by a letter and a number signifying the number of clones of that type (i.e. A21, is a MLG comprising 21 identical isolates). Percentage bootstrap values were generated from 1,000 replicates. Scale bar represents branch lengths. The three populations (I, II and III) inferred by Bayesian model-based analysis with program Structure 2.3.3 are circled and marked accordingly.
Fig. 4. *Trypanosoma evansi* isolates from Mindanao buffaloes classified into three populations I (blue), II (pink) and III (green) based on analysis of population structuring by the software program Structure 2.3.3. The size of the symbol reflects the number of isolates of that type collected from that location. The three provinces from which samples were collected and analysed were Agusan del Sur (ADS) and Surigao del Sur (SDS) and Agusan del Norte (ADN) (no buffalo isolates collected).
Fig. 5. Geographical representation of the occurrences of *Trypanosoma evansi* MLG A (●) and MLG C (●) isolates from buffalo in Mindanao, Philippines in relation to buffalo mortality figures (□). The size of the MLG symbols reflects number of isolates and the size of buffalo mortality symbol the buffalo mortality percentage. The three provinces from which samples were collected and analysed were Agusan del Sur (ADS) and Surigao del Sur (SDS) and Agusan del Norte (ADN) (no buffalo isolates collected). Major roads in provinces are marked. Arrows identify the location of MLG isolates which occurred in another province to all the other isolates of the respective MLG.