PHOENIX
ENVIRONMENTAL SCIENCES

The distribution of Aganippe 'MYG086' (Araneae: Idiopidae) in Western Australia

Prepared for BC Iron Ltd

September 2015

Final Report

The distribution of Aganippe 'MYG086' (Araneae: Idiopidae) in Western Australia

Prepared for BC Iron Ltd

Final Report

Author: Volker Framenau

Date: 23 September 2015

Submitted to: Les Purves, Michael Klvac

Chain of authorship and review			
Name	Task	Version	Date
Volker Framenau	Draft for technical review	1.0	6 May 2015
Jarrad Clark	Technical review	1.1	7 May 2015
Volker Framenau	Draft for client comments	1.2	8 May 2015
Volker Framenau	Final submitted to client	1.3	21 May 2015
Volker Framenau	Final submitted to client	2.0	23 September 2015

Phoenix Environmental Sciences Pty Ltd

1/511 Wanneroo Rd BALCATTA WA 6021

P: 08 9345 1608

F: 08 6313 0680

E: admin@phoenixenv.com.au

Project code: 1079-IV-BCI-TER

Contents

List of Tables

List of Appendices

EXECUTIVE SUMMARY

Aganippe 'MYG086' is currently considered a short-range endemic species (SRE) and only known from BC Iron Ltd's (BC Iron) Iron Valley Project and Roy Hill, approximately 80 km to the east. The presence of the species at Iron Valley resulted in restrictions in relation to vegetation clearing at the Iron Valley Project (Ministerial Statement 933, MS 933).

Phoenix Environmental Sciences (Phoenix) was commissioned by BC Iron to reassess the distribution of *Aganippe* 'MYG086' and its status as potential SRE.

Morphological identifications suggested that *Aganippe* 'MYG086' also occurs at Rosslyn Paroo Mine near Wiluna approximately 420 km south of Iron Valley. Subsequent molecular analyses (COI barcoding) showed COI sequence divergence values between specimens from Iron Valley and Rosslyn Paroo Mine to be less than 6.7% and therefore less than 9.5%, the current threshold value to differentiate most mygalomorph species.

Due to tissue degeneration, the analysed fragment (430 bp) was shorter than that used for a local barcoding study on mygalomorph spiders (656 bp). Therefore, a pairwise comparison of sequence divergences within Western Australian spiders in the family Idiopidae was conducted both for 452 bp and 658 bp fragments (total number of pairwise comparisons n = 2,340) to test the validity of the barcoding gap (9.5%) of the smaller fragment. This analysis showed clearly, that adding the missing data (206 bp) for *Aganippe* 'MYG086' is unlikely to change the divergence values based on the overall pattern of nucleotide variability in this fragment in the Western Australian Idiopidae. Adding data for any specimen pair with divergence under 10 % will add or reduce the divergence by generally less than 1% resulting in a maximum sequence divergence of 7.7% for the longer COI fragment of *Aganippe* 'MYG086' which remains well under the species-level cut-off value of 9.5%.

Therefore, molecular data cannot refute the hypothesis that populations of *Aganippe* 'MYG086' from Iron Valley and Rosslyn Paroo Mine established based on morphology belong to the same species. The distribution of the species spans at least three IBRA regions (Pilbara, Gascoyne, Murchison) and therefore it is not an SRE.

1 INTRODUCTION

Short-range endemics are organisms with a naturally narrow distribution range, in Western Australian nominally less than 10,000 km^2 (EPA 2009; Harvey 2002). There are uncertainties in determining the range-restrictions of many invertebrates in Western Australia due to lack of surveys, lack of taxonomic resolutions within target taxa and problems in identifying certain life stages. The WA Museum has introduced a three-tier system to account for these uncertainties, confirmed and potential SREs in addition to widespread species (Western Australian Museum 2013).

The Iron Valley Project in the Pilbara region of Western Australia received environmental approval pursuant to Ministerial Statement 933 (MS 933) (Minister for the Environment 2013). In relation to short-range endemic invertebrates, MS 933 states (p. 5):

- "*In order to ensure that the proposal does not result in significant impacts to the population size or distribution of Aganippe MYG086, the proponent shall ensure that no clearing or loss of vegetation occurs within the area of vegetation defined as Zone 1 in Figure 2 of Schedule 1, unless otherwise approved by the CEO under condition 8-2.*
- *Should the proponent demonstrate to the satisfaction of the CEO that Aganippe MYG086 is not a Short Range Endemic species, the proponent may apply to the CEO for approval to disturb vegetation within Zone 1.*"

These conditions were based on a short-range endemic (SRE) assessment conducted for the Project that found *Aganippe* 'MYG086' in the Project area but also about 80 km to the south-east (Dalcon 2012; Phoenix 2011).

The aim of this report is to re-evaluate the distribution of *Aganippe* 'MYG086' based on morphological and molecular analyses of specimens that became available since MS 933 was issued.

2 SCOPE OF WORKS

The key objective of the assessment is to investigate the SRE status of the potential SRE *Aganippe* 'MYG086'. Specifically, the scope comprises:

- an updated desktop review, in particular a search of the WA Museum Arachnology/Myriapodology database, to ascertain if new populations of *Aganippe* have been discovered since the 2010 and 2011 SRE assessments (Dalcon 2012; Phoenix 2011) have been conducted
- ascertaining the identification of unidentifiable historical records from these surveys using DNA sequencing against confirmed specimens, including potential *Aganippe* 'MYG086' recently collected near at Rosslyn Paroo Mine near Wiluna (Phoenix 2015).
- reassessing the SRE status and impact upon *Aganippe* within the project area.

3 METHODS

3.1 DESKTOP REVIEW

A database search for mygalomorph spiders in the family Idiopidae was requested from the WA Museum based on a square of 200 km length centred around Iron Valley and with the geographic coordinates (GDA94) -22.00°S/118.33°E (north-west corner) and 23.75°S/120.25°E (south-east

corner), consistent with the nominal ranges of SREs (i.e. 100 km x 100 km = 1,000 km^2) (EPA 2009; Harvey 2002).

3.2 MOLECULAR ANALYSES

The identification of species based on comparisons between DNA sequences is referred to as DNA barcoding. Any gene can be used for barcoding purposes; however, the primary gene targeted by researchers is Cytochrome Oxidase Subunit I (COI) (Hebert *et al.* 2003), which was targeted here. A molecular framework for mygalomorph spiders in relation to COI exists at the WA Museum (Castalanelli *et al.* 2014; Helix 2012).

DNA was amplified by utilising the molecular laboratory of the School of Animal Biology, University of Western Australia and amplified DNA was subsequently sequenced at the AGRF node Perth (Western Australian Institute of Medical Research, WA, Australia).

Six specimens were submitted for molecular analyses (Table 3-1). Standard primers (LCO/HCO) were used for amplification (Folmer *et al.* 1994), with the exception of WAM T114603 and T114604 (CI-j1718/HCO) (Hedin & Maddison 2001).

Following peer-review of the original analysis (Appendix 1) which queried the use of a shorter fragment of COI (430 bp) in comparison to Castalanelli *et al*.'s (2014) 658 bp, a pairwise comparison of sequence divergences for all Western Australian Idiopidae from Castalanelli *et al*.'s (2014) for both the short (452 bp) and the long (658 bp) fragment was conducted. This comparison aimed to explore if adding the missing data (206 bp) would significantly change the results of the original analysis (see Appendix 2 for details of the analysis).

Spider taxonomy follows the World Spider Catalog (2014).

All specimens, DNA extractions and sequence data will be submitted to the WA Museum.

Table 3-1 Specimens submitted for molecular analyses

WAM registration no.	Phoenix database no.	Locality	Latitude (GDA94)	Longitude (GDA94)	Sex
T114604	18703	Iron Valley	-22.4439778	119.18325	Male
T114603	18705	Iron Valley	-22.4437194	119.1837694	Male
T114602	18704	Iron Valley	-22.4338889	119.1958389	Female
T136800	17564	Rosslyn Paroo Mine, ca. 29.7 km WNW of Wiluna	-26.504225	119.9476333	Male
-[1]	17568	Rosslyn Paroo Mine, ca. 29.7 km WNW of Wiluna	-26.504225	119.9476333	Male
T136799	17574	Rosslyn Paroo Mine, ca. 29.7 km WNW of Wiluna	-26.504225	119.9476333	Male

1 – Reference specimen retained by Phoenix.

4 RESULTS

4.1 DESKTOP REVIEW

No specimens of *Aganippe* 'MYG086' were identified in the WA Museum database search within approximately 100 km Iron Valley in addition to the specimens identified in 2011. Further, no other specimens of *Aganippe* 'MYG086' were present in the WA Museum database outside the desktop research area (M. Castalanelli, pers. comm. to V.W. Framenau). However, the WA Museum database search identified at least 121 records of *Aganippe* species around Iron Valley in 10 species, five of which were identified by molecular methods (Table 4-1). These mainly represented females and could possibly be conspecific with *Aganippe* 'MYG086', of which COI sequences were not known prior to this study.

Table 4-1 *Aganippe* species identified by desktop review in vicinity of Iron Valley

Species	Number of records in desktop review area	Known sex	Remarks
Aganippe 'Cloudbreak sp. 1'	1	Male	
Aganippe 'MYG085'	14	Male, female	COI sequence known
Aganippe 'MYG086'	6	Male, female	Identification based on morphology
Aganippe 'MYG233'	3	Male, female	Identification based on morphology
Aganippe 'MYG300-DNA'	6	Female	Identified by COI sequence
Aganippe 'MYG303-DNA'	4	Female	Identified by COI sequence
Aganippe 'MYG305-DNA'	2	Female	Identified by COI sequence
Aganippe 'MYG306-DNA'	3	Female	Identified by COI sequence
Aganippe 'MYG384-DNA'	12	Unknown	Identified by COI sequence
Aganippe 'occidentalis?'	1	Male	
Unidentified *Aganippe*	69	Females, juveniles	
Total	**121**		

4.2 MOLECULAR ANALYSES

All specimens analysed provided usable DNA (although comparatively short fragments for the specimens from Iron Valley) and divergence analyses were conducted based on cropped alignments of 430 bp (full alignments in Table 4-2). COI barcoding confirmed the initial morphological identification of specimens from Rosslyn Paroo Mine as *Aganippe* 'MYG086' as the specimens did not differ by more than 6.7% (Table 4-3).

The pairwise comparison of smaller (452 bp) and larger (658 bp) fragments of COI divergences showed clearly, that adding the missing data for *Aganippe* 'MYG086' was extremely unlikely to change the divergence past the Castalanelli *et al.* (2014) working threshold of 9.5% (see Appendix 2) based on the overall pattern of nucleotide variability in this fragment in the Idiopidae. Adding data for any specimen pair with divergence under 10 % will add or reduce the divergence by generally less than 1%; in fact, the closer COI divergence approaches 10 %, adding more data will actually make the sequence divergence smaller (i.e. push it under 9.5%) (Appendix 2). This additional analysis was accepted as sufficient to confirm conspecifity of the population from Iron Valley and Rosslyn Paroo Mine by the initial reviewers of the original analysis (Appendix 3).

Comparing COI sequences of *Aganippe* 'MYG086' from Iron Valley with other *Aganippe* species from Western Australian lodged at the WA Museum and available on GenBank (Benson *et al.* 2012; Castalanelli *et al.* 2014) showed that *Aganippe* 'MYG086' is closest to *Aganippe '*MYG256' known from the Goldfields region of WA, but is clearly a distinct species (sequence divergence > 12.8%). *Aganippe* 'MYG086' is different to any other molecular species identified by the desktop review in the vicinity of Iron Valley.

Table 4-2 COI sequences (452–657 bp) of *Aganippe* 'MYG086' from Iron Valley and Rosslyn Paroo Mine used for divergence analysis

Specimen	COI sequence (cropped to 430 bp)
WAM T114602 (Iron Valley)	GATGTTAGGTGCACCTGATATAGCTTTTCCTCGTATAAATAATTTGAGATTTTGGTTGT TACCTCCTTCTTTGTTTTTTTTGATGGTCTCTTCGTTGGTTGAGGTGGGGGTTGGAGCT GGTTGGACTATTTATCCTCCTTTGTCTTCTGGTGTAGGGCATAGTGGGGGGGGAATGGA TTTTGTTATTTTTTCTTTACATTTAGCTGGGGCATCTTCGATTATGGGGGCTATTAATT TTATTTCTACTATTGTTAATATGCGTGCTGTAGGGATGGTGTTTGAACGTGTTTCTTTG TTTGTGTGGTCAGTGATAGTGACGGCTGTGTTGCTTTTGTTGTCTCTTCCTGTGTTGGC GGGTGCTATTACAATATTATTGACTGATCGAAATTTTAATACTTCTTTTTTTGACCCGG CTGGGGGTGGGGATCCTGTTTTATTTCAGCATTTATTTT
WAM T114604 (Iron Valley)	GATGTTAGGTGCACCTGATATAGCTTTTCCTCGTATAAATAATTTGAGATTTTGGTTGT TACCTCCTTCTTTGTTTTTTTTGATGGTCTCTTCGTTGGTTGAGGTGGGGGTTGGAGCT GGTTGGACTATTTATCCTCCTTTGTCTTCTGGTGTAGGGCATAGTGGGGGGGGAATGGA TTTTGTTATTTTTTCTTTACATTTAGCTGGGGCATCTTCGATTATGGGGGCTATTAATT TTATTTCTACTATTGTTAATATGCGTGCTGTAGGGATGGTGTTTGAACGTGTTTCTTTG TTTGTGTGGTCAGTGATAGTGACGGCTGTGTTGCTTTTGTTGTCTCTTCCTGTGTTGGC GGGTGCTATTACAATATTATTGACTGATCGAAATTTTAATACTTCTTTTTTTGACCCGG CTGGGGGTGGGGATCCTGTTTTATTTCAGCATTTATTTT
WAM T114603 (Iron Valley)	AGTTCCTTTGATGTTAGGTGCACCTGATATAGCTTTTCCTCGTATAAATAATTTGAGAT TTTGGTTGTTACCTCCTTCTTTGTTTTTTTTGATGGTCTCTTCGTTGGTTGAGGTGGGG GTTGGAGCTGGTTGGACTATTTATCCTCCTTTGTCTTCTGGTGTAGGGCATAGTGGGGG GGGAATGGATTTTGTTATTTTTTCTTTACATTTAGCTGGGGCATCTTCGATTATGGGGG CTATTAATTTTATTTCTACTATTGTTAATATGCGTGCTGTAGGGATGGTGTTTGAACGT GTTTCTTTGTTTGTGTGGTCAGTGATAGTGACGGCTGTGTTGCTTTTGTTGTCTCTTCC TGTGTTGGCGGGTGCTATTACAATATTATTGACTGATCGAAATTTTAATACTTCTTTTT TTGACCCGGCTGGGGGTGGGGATCCTGTTTTATTTCAGCATTTATTTT

Specimen	COI sequence (cropped to 430 bp)
WAM T136799 (Rosslyn Paroo Mine)	CTATATATATTATTTTGGGTGTGTGGTCGGCGATATTAGGAACAGCAATAAGAGTAGTG ATTCGGACAGAGTTGGGGCAAGTTGGAAGTCTTTTAGGAAATGATCATTTGTATAATGT AGTTGTGACGGTTCATGCTTTAGTTATAATTTTTTTTATGGTGATGCCTATTATAATTG GAGGGTTTGGAAATTGATTGGTACCTTTGATGTTAGGGTCGCCGGATATGGCTTTTCCT CGTATAAATAATTTGAGATTTTGGTTGTTGCCTCCTTCTTTATTTTTTTTAATGGTTTC TTCGTTAGTTGATGTGGGAGTTGGGGCTGGTTGGACTATTTATCCTCCTTTGTCTTCTA GTATAGGTCATAGAGGGGGGGGAATGGATTTTGTTATTTTTTCTTTACATTTAGCTGGT GCATCTTCGATTATGGGAGCTATTAATTTTATTTCTACTATTGTGAATATGCGAGCTGT GGGGATGGTGTTTGAGCGTGTTTCTTTGTTTGTGTGGTCAGTGATGGTGACAGCTGTAT TACTGTTGTTGTCTCTTCCTGTGTTGGCGGGGGCTATCACAATATTATTGACTGATCGA AATTTTAATACTTCCTTTTTCGACCCGGCTGGGGGAGGGGATCCTGTTTTATTTCAACA TTTATTTT
Phoenix 17568 (Rosslyn Paroo Mine)	CTATATATATTATTTTGGGTGTGTGGTCGGCGATATTAGGAACAGCAATAAGAGTAGTG ATTCGGACAGAGTTGGGGCAAGTTGGAAGTCTTTTAGGAAATGATCATTTGTATAATGT AGTTGTGACGGTTCATGCTTTAGTTATAATTTTTTTTATGGTGATGCCTATTATAATTG GAGGGTTTGGAAATTGATTGGTACCTTTGATGTTAGGGTCGCCGGATATGGCTTTTCCT CGTATAAATAATTTGAGATTTTGGTTGTTGCCTCCTTCTTTATTTTTTTTAATGGTTTC TTCGTTAGTTGATGTGGGAGTTGGGGCTGGTTGGACTATTTATCCTCCTTTGTCTTCTA GTATAGGTCATAGAGGGGGGGGAATGGATTTTGTTATTTTTTCTTTACATTTAGCTGGT GCATCTTCGATTATGGGAGCTATTAATTTTATTTCTACTATTGTGAATATGCGAGCTGT GGGGATGGTGTTTGAGCGTGTTTCTTTGTTTGTGTGGTCAGTGATGGTGACAGCTGTAT TACTGTTGTTGTCTCTTCCTGTGTTGGCGGGGGCTATCACAATATTATTGACTGATCGA AATTTTAATACTTCCTTTTTCGACCCGGCTGGGGGAGGGGATCCTGTTTTATTTCAACA TTTATTTT
WAM T136800 (Rosslyn Paroo Mine)	CTATATATATTATTTTGGGTGTGTGGTCGGCGATATTAGGAACAGCAATAAGAGTAGTG ATTCGGACAGAGTTGGGGCAAGTTGGAAGTCTTTTAGGAAATGATCATTTGTATAATGT AGTTGTGACGGTTCATGCTTTAGTTATAATTTTTTTTATGGTGATGCCTATTATAATTG GAGGGTTTGGAAATTGATTGGTACCTTTGATGTTAGGGTCGCCGGATATGGCTTTTCCT CGTATAAATAATTTGAGATTTTGGTTGTTGCCTCCTTCTTTATTTTTTTTAATGGTTTC TTCGTTAGTTGATGTGGGAGTTGGGGCTGGTTGGACTATTTATCCTCCTTTGTCTTCTA GTATAGGTCATAGAGGGGGGGGAATGGATTTTGTTATTTTTTCTTTACATTTAGCTGGT GCATCTTCGATTATGGGAGCTATTAATTTTATTTCTACTATTGTGAATATGCGAGCTGT GGGGATGGTGTTTGAGCGTGTTTCTTTGTTTGTGTGGTCAGTGATGGTGACAGCTGTAT TACTGTTGTTGTCTCTTCCTGTGTTGGCGGGGGCTATCACAATATTATTGACTGATCGA AATTTTAATACTTCCTTTTTCGACCCGGCTGGGGGAGGGGATCCTGTTTTATTTCAACA TTTATTTT

Table 4-3 COI sequence divergence (upper right half) and congruence (lower bottom half) of *Aganippe* 'MYG086' from Iron Valley and Rosslyn Paroo Mine (COI sequence length = 430bp)

Location	Registration no.	WAM T136800	Phoenix 17568	WAM T136799	WAM T114604	WAM T114602	WAM T114603
Rosslyn Paroo Mine	WAM T136800		0	0	6.7	6.7	6.7
	Phoenix 17568	100		0	6.7	6.7	6.7
	WAM T136799	100	100		6.7	6.7	6.7
Iron Valley	WAM T114604	93.3	93.3	93.3		0	0
	WAM T114602	93.3	93.3	93.3	100		0
	WAM T114603	93.3	93.3	93.3	100	100	

5 DISCUSSION

Species identification based on COI barcoding is not without problems as sequence divergence within species can be high and may exceed that between species in some taxa, including SRE target groups (e. g. Bond 2004; Boyer *et al.* 2007; Köhler & Johnson 2012). A comprehensive study of WA mygalomorph spiders focussing on species from the Pilbara region identified a sequence divergence of approximately 9.5% as proposed cut-off value to differentiate separate species (Castalanelli *et al.* 2014). Based on this study, with a maximum divergence of 6.7% the specimens of *Aganippe* 'MYG086' from Iron Valley and Rosslyn Paroo Mine are unequivocally the same species, in particular taking the large distance of the two populations into account. This result also holds true considering the comparatively short length of the COI fragment analysed (Appendices 1–3).

With a distance of more than 420 km between currently known records of *Aganippe* 'MYG086', the known distribution of the species far exceeds what is currently used as nominal or working range for short-range endemism, i.e. 10,000 km^2 (= 100 x 100 km^2). The distribution of the species spans at least three IBRA bioregions, Pilbara, Gascoyne and Murchison (Department of the Environment 2015). *Aganippe* 'MYG086' appears to reach the northern limits of its distribution at Iron Valley considering the good survey density in the eastern Pilbara and is therefore probably only known from a few localities. Considering the poor collection coverage in the Gascoyne and Murchison region, it is very likely that *Aganippe* 'MYG086' will be found at many more localities throughout these regions.

In summary, *Aganippe* 'MYG086' is not a short-range endemic species and it is suggested to re-evaluate the conditions of MS 933 in relation to this species.

6 REFERENCES

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D., Ostell, J. & Sayers, E. W. 2012. GenBank. *Nucleic Acids Research* **41**: D36–D42.

Bond, J. E. 2004. Systematics of the Californian euctenizine spider genus *Apomastus* (Araneae: Mygalomorphae: Cyrtaucheniidae): the relationship between molecular and morphological taxonomy. *Invertebrate Systematics* **18**: 361–376.

Boyer, S. L., Baker, J. M. & Giribet, G. 2007. Deep genetic divergences in *Aoraki denticulata* (Arachnida, Opiliones, Cyphophthalmi): a widespread 'mite harvestman' defies DNA taxonomy. *Molecular Ecology* **16**: 4999–5016.

Castalanelli, M. A., Teale, R., Rix, M. G., Kennington, J. & Harvey, M. S. 2014. Barcoding of mygalomorph spiders (Araneae: Mygalomorphae) in the Pilbara region of Western Australia *Invertebrate Systematics* **28**: 375–385.

Dalcon. 2012. *Iron Ore Holding Ltd - Iron Valley Project. Targeted terrestrial short-range endemic invertebrate fauna survey*. Dalcon Environmental Pty Ltd, Malaga, WA. Unpublished report prepared for URS Australia Pty Ltd.

Department of the Environment. 2015. *Maps: Australia's bioregions (IBRA)*. Department of the Environment, Canberra, ACT. Available at: http://www.environment.gov.au/topics/land/national-reserve-system/science-maps-and-data/australias-bioregions-ibra (accessed 24 January 2015).

EPA. 2009. *Guidance for the assessment of environmental factors (in accordance with the Environmental Protection Act 1986). Sampling of short range endemic invertebrate fauna for environmental impact assessment in Western Australia. No. 20*. Environmental Protection Authority, Perth, WA. Available at:

http://www.epa.wa.gov.au/EPADocLib/2953_GS20SRE250509.pdf (accessed 7 September 2012).

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. C. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294–299.

Harvey, M. S. 2002. Short-range endemism among the Australian fauna: some examples from non-marine environments. *Invertebrate Systematics* **16**: 555–570.

Hebert, P. D. N., Ratnasingham, S. & de Waard, J. R. 2003. Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society London B, Supplement* **270**: 96–99.

Hedin, M. C. & Maddison, W. P. 2001. A combined molecular approach to phylogeny of the jumping spider subfamily Dendryphantinae (Araneae, Salticidae). *Molecular Phylogenetics and Evolution* **18**: 386–403.

Helix. 2012. *Report on the molecular systematics of Mygalomorphae from the Pilbara*. Helix Molecular Solutions, School of Animal Biology, The University of Western Australia, Crawley, W.A. Unpublished report to Biota Environmenal Science Pty Ltd.

Köhler, F. & Johnson, M. S. 2012. Species limits in molecular phylogenies: a cautionary tale from Australian land snails (Camaenidae: *Amplirhagada* Iredale, 1933). *Zoological Journal of the Linnean Society* **165**: 337–362.

Minister for the Environment. 2013. *Statement that a proposal may be implemented (pursuant to the provisions of the Environmental Protection Act 1986). Iron Valley Above Watertable Mining Project. Iron Ore Holdings Ltd*. Western Australia Minister for the Environment, Perth, WA. Unpublished statement to Iron Ore Holding Ltd.

Phoenix. 2011. *Trapdoor spiders (Araneae: Mygalomorphae) from Iron Valley, near Marillana Station*. Phoenix Environmental Sciences Pty Ltd, Balcatta, WA. Unpublished draft report prepared for Dalcon Environmental.

Phoenix. 2015. *Identification and assessment of short-range endemism of invertebrates from Rosslyn Paroo Mine Survey, Western Australia*. Phoenix Environmental Sciences Pty Ltd, Balcatta, WA. Unpublished report prepared for Dalcon Environmental Pty Ltd.

Western Australian Museum. 2013. *WAM short-range endemic categories*. Western Australian Museum, Welshpool, WA.

World Spider Catalog. 2014. *World Spider Catalog*. Natural History Museum Bern, Bern (Switzerland). Available at: http://wsc.nmbe.ch/ (accessed 26 August 2014).

Appendix 1: Peer-review of original report by Drs M.S. Harvey and M.R. Rix (WA Museum)

(see next page)

25 August 2015

Mr Les Purves
Environmental Adviser
BC Pilbara Iron Ore Pty Ltd
GPO Box 2811
West Perth WA 6872

Re: Report by Phoenix Environmental Sciences (May 2015) "The distribution of *Aganippe* 'MYG086' (Araneae: Idiopidae) in Western Australia"

Dear Mr Purves

In response to your request for a peer review of the above report by Phoenix Environmental Sciences, we have prepared a short summary (below). This review is focused on the molecular conclusions presented in the report, and we have applied the same rigour that we would to a scholarly journal article. We hope it satisfies the conditions of the Office of the Environmental Protection Authority (OEPA).

Please let us know if you require any further information or clarification, and with our very best wishes,

Mark Harvey and Mike Rix
Department of Terrestrial Zoology

WESTERN AUSTRALIAN MUSEUM
Locked Bag 49, Welshpool DC, WA 6986, AUSTRALIA
Telephone: +61 8 9212 3737 • Facsimile: +61 8 9212 3882
Web: www.museum.wa.gov.au • email mark.harvey@museum.wa.gov.au
PERTH • MARITIME • FREMANTLE • GERALDTON • KALGOORLIE-BOULDER • ALBANY

Report by Phoenix Environmental Sciences (May 2015) "The distribution of *Aganippe* 'MYG086' (Araneae: Idiopidae) in Western Australia"

Summary Review

This report tests the distribution of the idiopid trapdoor spider *Aganippe* sp. 'MYG086', previously known from the Iron Valley Project and Roy Hill, Western Australia. The primary objectives of the work contained in Phoenix (2015) were to test – using molecular data – whether: (A) specimens from Rosslyn Paroo Mine near Wiluna were conspecific with those from Iron Valley/Roy Hill; and thus (B) whether 'MYG086' can be considered a short-range endemic species. For the purposes of this review we comment solely on the conclusions made using the molecular dataset presented in Phoenix (2015), and whether our analyses of those data match the results presented in the report.

Table 1. Sequence lengths for the *CO1* sequences presented in Phoenix (2015).

Specimen	Published sequence length (bp)
WAM T114602 (Iron Valley)	452
WAM T114604 (Iron Valley)	452
WAM T114603 (Iron Valley)	461
WAM T136799 (R. Paroo Mine)	657
Phoenix 17568 (R. Paroo Mine)	657
WAM T136800 (R. Paroo Mine)	657

In assessing the molecular results presented in Phoenix (2015), we copied the six published *CO1* sequences from pages 4-5 of the report and analysed them using Geneious 6.1.7. Phoenix (2015: 4) stated that "analyses were conducted based on cropped alignments of 430 bp". However, three of the sequences presented were not in fact cropped below the length of the standard barcoding segment (which is 658 bp), and none were 430 bp in length, as stated. A summary of the published sequence lengths is shown in Table 1 (above).

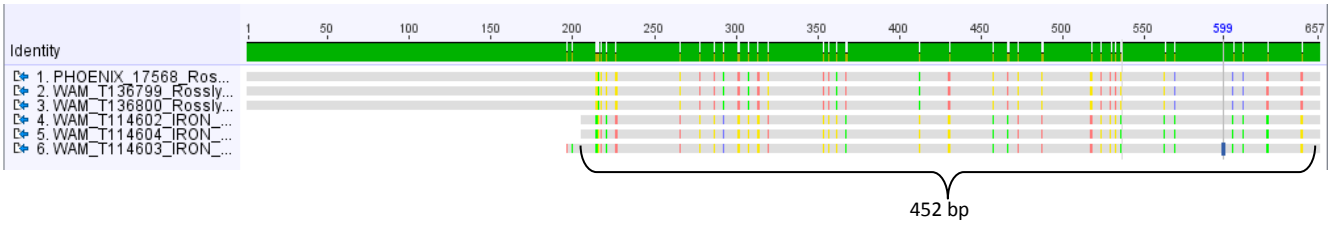

Figure 1. Graphical alignment of the six *CO1* sequences presented in Phoenix (2015), highlighting the 452 bp overlapping region of the barcoding segment.

After alignment of these six sequences in Geneious, a core overlapping region of 452 bp was recovered (see Fig. 1). We therefore cropped the alignment to 452 bp accordingly, and analysed the pairwise divergence values among taxa. These values are summarised in Table 2, in an identical format to that presented in Table 4-3 of Phoenix (2015).

Table 2. *CO1* sequence divergence (upper right half) and congruence (lower bottom half) of *Aganippe* 'MYG086' from Iron Valley and Rosslyn Paroo Mine (*CO1* sequence length = **452 bp**). Differences from Phoenix (2015) are highlighted in red.

Location	Registration no.	WAM T136800	Phoenix 17568	WAM T136799	WAM T114604	WAM T114602	WAM T114603
	WAM T136800		0	0	7.5	7.5	7.5
Rosslyn Paroo Mine	Phoenix 17568	100		0	7.5	7.5	7.5
	WAM T136799	100	100		7.5	7.5	7.5
	WAM T114604	92.5	92.5	92.5		0	0
Iron Valley	WAM T114602	92.5	92.5	92.5	100		0
	WAM T114603	92.5	92.5	92.5	100	100	

Conclusions

In short, we recovered different results to those reported in Phoenix (2015), namely that pairwise divergence values between populations of *Aganippe* 'MYG086' from Rosslyn Paroo Mine vs. Iron Valley were higher than previously reported (i.e. 7.5% cf. 6.7%). Our analysed portion was also 22 bp larger than that reported in Phoenix (2015), and this is no doubt the source of the discrepancy. Indeed, if the first 22 bp of our alignment is cropped, then the results presented in Phoenix (2015) are obtained. However, we can see no compelling reason for cropping these data beyond 452 bp – and certainly no reason was given in Phoenix (2015) – especially given that the fragment is already considerably shorter than is generally required for accurate *CO1* barcoding. The cropping of 22 bp by Phoenix (2015) seems further problematic, as there are five variable nucleotide sites in this region which change the results by nearly a percentage point (see Table 2).

In summary, we can conclude that while up to 7.5% *CO1* sequence divergence is still within the threshold that we currently understand to represent conspecific taxa, this value is different (higher) than that presented by Phoenix (2015), due to the latter inexplicably removing usable data (i.e. 22 bp). We also interpret these results with great caution, as the 9.5% divergence conspecific cut-off value suggested by Castalanelli et al. (2014) was based on analysis of the full 658 bp barcoding fragment, and not a highly reduced 452 bp fragment as presented here (see below).

Recommendations

Our recommendations are as follows:

(A) The three specimens from Iron Valley should be re-sequenced, so that the entire barcoding fragment is recovered for these taxa; and

(B) Pairwise divergence calculations should be made using the full-length barcoding fragment of 658 bp, so that the 9.5% threshold can be applied accurately as per Castalanelli et al. (2015). Indeed, as the addition of 22 bp can significantly change a divergence result, the addition of a further 206 bp could likewise alter the result and therefore change one's interpretation.

References

Castalanelli, M.A., Teale, R., Rix, M.G., Kennington, W.J. and Harvey, M.S. (2014). Barcoding of mygalomorph spiders (Araneae : Mygalomorphae) in the Pilbara bioregion of Western Australia reveals a highly diverse biota. *Invertebrate Systematics* **28,** 375-385.

Phoenix (2015). The distribution of *Aganippe* 'MYG086' (Araneae: Idiopidae) in Western Australia. Report prepared for BC iron Ltd (May 2015), pp.1-7.

Mark Harvey and Mike Rix
Western Australian Museum
24 August 2015

Appendix 2: Reply by Phoenix Environmental Sciences to peer-review by Drs M.S. Harvey and M.R. Rix (WA Museum)

(see next page)

Memo

To: Michael Rix, Mark Harvey

From: Volker Framenau

CC:

Date: 31 August 2015

Subject: Review of Phoenix report on *Aganippe* 'MYG086'

Hi Mark and Mike,

Thank you very much for taking the time to review our report on barcoding *Aganippe* 'MYG086' specimens for our client BC Iron. Les Purves has asked me to have a look at your review and recommend further action, ideally avoiding further molecular work due to time and resources constraints.

We have critically reviewed you document and have a number of comments to make, both in regard to our initial analyses and your review of it, but also in relation to your recommendation to repeat the molecular work to obtain a larger COI sequence, ideally a 'standard fragment' of 658 bp. We are happy to revise our initial document with the details below which in our opinion is sufficient to show that there is not much evidence to support that the specimens analysed represent more than one species.

Overall, our primary test was not, as stated in your review, to "test whether the specimens were conspecific", but to potentially reject the null-hypothesis that they are conspecific, which was established by morphological examination before. In other words, molecular data were used to potentially disprove morphological conclusions that there was little difference between the specimens examined and they represent a single morphospecies. All specimens are morphological indistinguishable in relation to male pedipalp appendices or somatic characters which only initiated the analysis in the first place. In my opinion and based on the examination of many *Aganippe* males, the genus has very reliable genitalic characters represented by a variation of apophysis on the pedipalp and high inter-specific morphological variability in the embolus, much unlike, for example, in the Nemesiidae. Of course, that would not exclude cryptic speciation, but as argued below, molecular data will very likely fail to disprove conspecifity, even if we add further nucleotide sequences.

Why short fragment of specimens from Iron Valley?

The specimens from Iron Valley were collected in 2011 (fairly old and dubious tissue preparation at Dalcon) and using standard primers/PCRs did not yield longer fragments than 452 bp. Trial PCRs run using both Folmer standard primers (resulting in amplicon lengths of ca. 650 bp) and an additional reverse primer for longer fragments (820 bp) failed to amplify DNA, presumably because the specimens are old and the initial preservation of the specimens was not optimal.

There are two reasons why we only analysed 430 bp of these. :

- The initial analysis included a further unrelated specimen with 430 bp which was omitted in the report. As there was no overall change in the result for 452 bp (i.e. divergence values of *Aganippe* 'MYG086' much lower than current operational threshold), there was no need to redo the analysis.
- We preferred cropping the sequences of 452 bp to 430 bp because the initial 22 bp were close to forward primer sequence and we find that the region that flank primer sequences are often 'messy' and can produces false and/or dubious base call in the chromatogram. We prefer cropping the 5' and 3' ends of the DNA sequences rigorously to avoid inconsistency in molecular data collection. The necessity to do this was quite nicely shown by your analysis of the 452 bp sequences. An increase of a disproportionate 0.8% (from 6.7% to 7.5%) caused by five variable nucleotides when adding only 22 bp to the analysis appears to be caused by

false reads near the primer site (see our analysis of variability in the missing sequence below).

Within this context, the heading of our Table 4-2 ("Cropped (430 bp) COI sequence…") is an error that was not picked up in our internal review process.

Will adding further data to the dataset increase COI divergence?

The recommendation to re-sequence the Iron Ore Valley specimens to obtain longer sequences makes only sense if there is evidence that the variability in the missing 206 bp is disproportionally higher than in the 430 or 452 bp fragments that have been analysed. No evidence, either from published papers or analyses of data, is put forward in the review with the exception of the high nucleotide variability between 430 and 458 bp which, however, may be explained by false reads. We also stress that, even if the reads were true, the modified divergence values of 7.5 per cent between the sequenced samples I still below the Hebert threshed of 8 per cent for arachnid species that is commonly used, and certainly below the threshold you recently proposed for WA mygalomorph species (Castalanelli *et al.* 2014) (9.5 per cent; with often higher values intrinsically applied if the underlying data in this paper are checked carefully).

Scientifically, there is no accepted minimum length of primers for COI barcoding, nor a standard divergence threshold that defines species. Indeed, many published analyses of barcoding in spiders use much smaller fragments than the 658 bp analysed by Castalanelli *et al.* (2014), for example 420 bp in Robinson *et al.* (2009), and others use larger sequences (e.g. Lopardo & Uhl 2014). The Consortium for the Barcode of Life defines the 'Folmer region' as standard, which includes 648 bp (http://www.barcodeoflife.org/content/resources/standards-and-guidelines). They also recommend to use a least 75% contiguous, high quality bases of this sequence (i.e. 486 bp), which is only about 10% more than what we analysed.

Evidently, the most appropriate way to gather some evidence of COI divergence patterns in the missing fragment from the taxon at hand, the genus *Aganippe* or, as there are doubts on current generic limits of this genus, the family Idiopidae, is to analyse the taxon specific data. We have therefore simply re-analysed the idiopid dataset of Castalanelli *et al.* (2014) and calculated COI divergences of all specimen pairs (n = 2,340) of 81 specimens of Idiopidae for 658 bp and 450 bp and plotted a pairwise comparison of these divergence differences (Figure 1). Data for specimen pairs of specimens currently identified as *Aganippe* (30 specimens, 435 species pairs) are also illustrated and of course simple represent a subset of the whole dataset without changing the overall result (Figure 1).

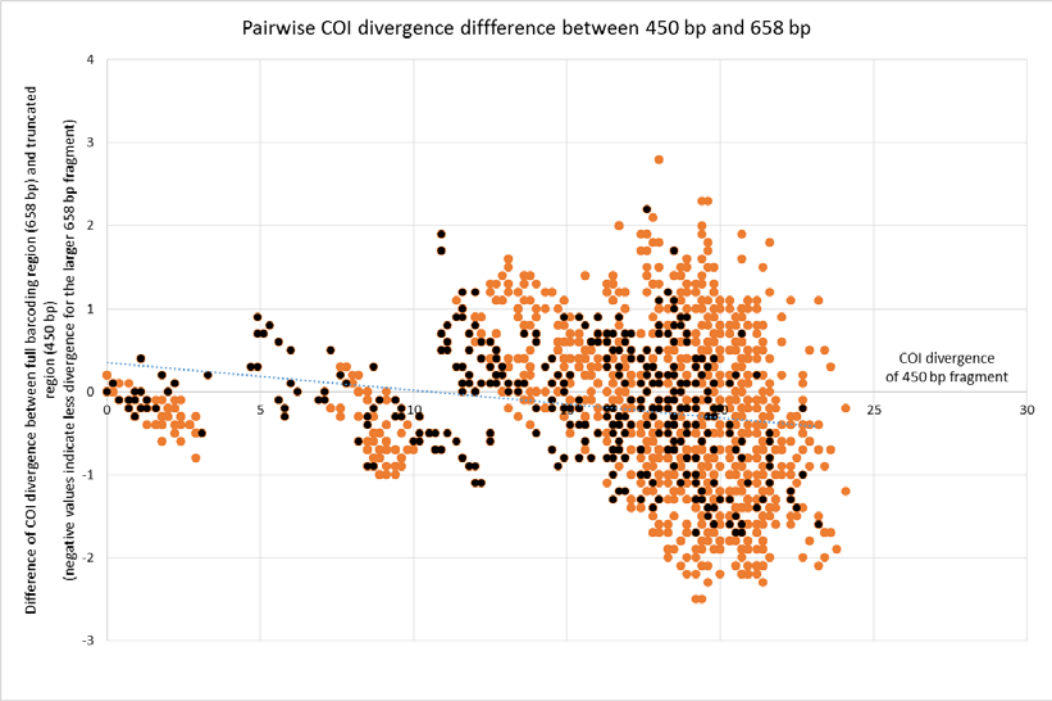

Figure 1: Pairwise differences of COI sequence divergence between 658 bp and 450 bp in relation to COI divergence of the 450 bp fragment for WA *Aganippe* (black dots) or Idiopidae (orange dots) (data from Castalanelli *et al.* 2014)

This analysis shows clearly, that adding the missing data for *Aganippe* 'MYG086' is extremely unlikely to change the divergence over the Castalanelli *et al.* (2014) working threshold of 9.5% (here corroborated as a gap in the scatterplot between ca. 9.5 and 12 %) based on the overall pattern of nucleotide variability in this fragment in the Idiopidae. Adding data for any specimen pair with divergence under 10 % will add or reduce the divergence by generally less than 1%; in fact, the closer COI divergence approaches 10 %, adding more data will actually make the sequence divergence smaller (i.e. push it under 9.5%) as indicated be the differences tending to < 0. In other words, even if we accept that 7.5% is the true divergence at 452bp, adding 206 bp is very unlikely to change the divergence to more than 8.5%, in fact, it is just as likely to reduce it to 6.5%.

In summary, there is currently now scientific evidence to support the position that adding a further 206 bp to the analysis will increase the COI divergence between the specimens collected at Iron Valley and Paroo Rosslyn Mine above the current species level cut-off of 9.5% in the WA Idiopidae. The current null-hypothesis based on morphology is not being refuted by molecular data and, following scientific rigour applied to the data currently available (morphology and molecular), the specimen should be considered the same species.

Best regards,

Volker Framenau

Director; Manager, Invertebrate Sciences

volker.framenau@phoenixenv.com.au
08 9345 1608 (t)
0418 203 320 (m)
1/511 Wanneroo Road, Balcatta WA 6021

References

Castalanelli, M. A., Teale, R., Rix, M. G., Kennington, J. & Harvey, M. S. 2014. Barcoding of mygalomorph spiders (Araneae: Mygalomorphae) in the Pilbara region of Western Australia *Invertebrate Systematics* **28**: 375–385.

Lopardo, L. & Uhl, G. 2014. Testing mitochondrial marker efficacy for DNA barcoding in spiders: a test case using the dwarf spider genus *Oedothorax* (Araneae : Linyphiidae : Erigoninae). *Invertebrate Systematics* **28**: 501–521.

Robinson, E. A., Blagoev, G. A., Hebert, P. D. N. & Adamowicz, S. J. 2009. Prospects for using DNA barcoding to identify spiders in species-rich genera. *ZooKeys* **16**: 27–46.

Appendix 3: Acceptance of reply to peer-review by WA Museum

(see next page)

From: Mark Harvey [mailto:Mark.Harvey@museum.wa.gov.au]
Sent: 18 September, 2015 9:46 AM
To: Volker Framenau <volker.framenau@phoenixenv.com.au>
Cc: Michael Rix <Michael.Rix@museum.wa.gov.au>
Subject: RE: Aganippe barcoding

Dear Volker

We have read your email and memo, and are happy to provide the following statement, which you can send to Les with your revised report:

Statement

We have read the addendum provided by Phoenix Environmental Sciences in relation to the Report on *Aganippe* 'MYG086', and agree with the findings therein. We consider this to be a scientifically appropriate and rigorous response to our concerns surrounding the use of only 450 bp of CO1. Our only major recommendation going forward would be that if Castalanelli et al. (2014) is used as a benchmark for hypothesis-testing, the reasoning behind using shorter fragments should be explicitly stated and justified in the first instance (as was done subsequently).

In summary, we agree with the conclusion that:
"*In summary, there is currently now scientific evidence to support the position that adding a further 206 bp to the analysis will increase the COI divergence between the specimens collected at Iron Valley and Paroo Rosslyn Mine above the current species level cut-off of 9.5% in the WA Idiopidae. The current null-hypothesis based on morphology is not being refuted by molecular data and, following scientific rigour applied to the data currently available (morphology and molecular), the specimen should be considered the same species.*"

We thus endorse the findings of the initial report and the supplied addendum, in combination.

Best wishes

Mark Harvey & Mike Rix

.....
Dr Mark S. Harvey
Senior Curator & Head, Dept. of Terrestrial Zoology
Western Australian Museum, Locked Bag 49, Welshpool DC, WA 6986, AUSTRALIA
Phone + 61 8 9212 3737, Fax + 61 8 9212 3882
Email: mark.harvey@museum.wa.gov.au
.....
Adjunct Professor, University of Western Australia
Research Associate, American Museum of Natural History
Research Associate, California Academy of Sciences
.....