

Allegory of a cave crustacean: systematic and biogeographic reality of *Halosbaena* (Peracarida: Thermosbaenacea) sought with molecular data at multiple scales

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Received: 14 January 2016 / Revised: 21 August 2016 / Accepted: 29 August 2016
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Abstract *Halosbaena* Stock, 1976 are small crustaceans found in a number of distant, isolated subterranean locations in the Northern (Caribbean and Canary Islands) and Southern Hemispheres (Christmas Island and north-western Australia in Cape Range, Barrow Island and Pilbara regions). This distribution is surprising for an animal that produces few eggs, has no free-living larval stage, and succours their young in a dorsal brood pouch. It is usually explained by the passive movement of ancestral populations on tectonic plates as the ancient Tethys Ocean spread. We used molecular data (one mitochondrial and three nuclear genes) to reconstruct phylogenies and time-trees to understand their biogeography at the global scale and at four diminishing scales within the Southern Hemisphere. We found: (1) a basal split between species in the Northern and Southern Hemispheres, but the inferred ages of divergences between species are not old enough to be associated with the spread of the Tethys; (2) a recently discovered species from Christmas Island which is the sister to Australian mainland taxa; (3) the one described species from

mainland Australia, *H. tulki*, probably constitutes at least five separate species that reflect local geography (Cape Range west, Cape Range east, Barrow Island, Pilbara low elevation, Pilbara high elevation); (4) the Pilbara high elevation taxon is likely not old enough to have been stranded high inland during an Eocene marine transgression; and (5) phylogeographic breaks within Cape Range west are congruent with other breaks in sympatric cave species.

Keywords Anchialine · Australia · Cryptic species · Phylogeny · Stygofauna · Subterranean · Tethys

Introduction

Plato presented the search for the truth underlying superficial appearances as being like the relationship between shadows seen on a metaphorical cave wall and the real objects that have cast them (Winsor 2006). The distribution of cave animals (the

Communicated by F. Leese

Electronic supplementary material The online version of this article (doi:10.1007/s12526-016-0565-3) contains supplementary material, which is available to authorized users.

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“shadows”) has inspired wide-ranging biogeographic and evolutionary theories that attempt to explain the underlying processes (the “objects”) responsible for creating the observed patterns (Juan et al. 2010). This is similar to the way in which oceanic islands have often provided a key to unlocking the workings of evolution (Cooper et al. 2007). Subterranean life is particularly suited to similar investigations, since it is frequently limited to very specific and restricted, island-like, environments, and so underlying tectonic forces may provide an explanatory narrative for both the landscape and biota.

One such subterranean group that has generated much interest is the Thermosbaenacea Monod, 1927, which is a small order of shrimp-like, peracarid crustaceans. Typical of stygofauna (aquatic subterranean animals), they are small (<4 mm), lack eyes and pigment, and inhabit groundwater of variable salinity (fresh to marine) in caves, springs and alluvia (Jaume 2008). They are especially associated with anchialine ecosystems, which are subterranean estuaries (Bishop et al. 2015) of open water or groundwater on the coast under the influence of marine tides through subterranean connections to the sea, often with a marked halocline (Sket 1996). There are seven recognised thermosbaenacean genera, which produce few eggs, have no free-living larval stage, and, uniquely amongst peracarids, females succour their young in a dorsal rather than ventral brood pouch (Jaume 2008). Given these factors, thermosbaenaceans are assumed to have little capacity for dispersal (Wagner 1994). Obligate inhabitants of the underworld, both aquatic and terrestrial, have been considered trapped by their biology in subterranean spaces, after which their biogeography is likely at the whim of geological rather than biological processes (Humphreys 2006).

However, these tiny, blind cave crustaceans have a distribution that would be the envy of a large seabird, with one genus, *Halosbaena* Stock, 1976, being found at locations spanning 20,000 km, which is about as far apart as any two points can be on the surface of the Earth (Fig. 1). This curious pattern has fostered numerous theories. Foremost amongst these is the supposition that the distribution of the Thermosbaenacea was moulded by the ancient Tethys Sea (Jaume 2008) (see Fig. 2). This is because the recent geographic extent of the order, namely the Caribbean, Mediterranean, Canary Islands, north Western Australia (NWA) and Southeast Asia (the so-called “Full Tethyan track”; Heads 2013; Stock 1993) mirrors the shores of this former sea, girt by continental blocks, which once extended halfway around the world during the Mesozoic (252–66 million years ago). The Tethys was an epicontinental ocean that, during the dispersal of Pangaea in the Jurassic, spread westward between Gondwana and Laurasia into the proto-Atlantic Ocean. The North Atlantic deepened between 110 and 95 million years ago (Jones et al. 1995; Sclater et al. 1977) forming a potential barrier to shallow water dispersal. Marine faunal interchange between the eastern Atlantic and

Indo-West Pacific regions could have occurred along the Tethyan Seaway between the Mediterranean and Arabian Seas until at least 19 MYA (million years ago) (Harzhauser et al. 2007), and possibly as recently as 10 MYA (McQuarrie et al. 2003).

The basic idea behind the influence of the Tethys is that thermosbaenacean ancestors lived in shallow, near-shore environments (perhaps marine caves, and progressively more anchialine areas), and were transported to their current, distant locations passively via the movement of tectonic plates as the Tethys spread (Cals and Monod 1988; Jaume 2008; Maquire 1965; Monod and Cals 1988; Stock 1976; Wagner 1990, 1994). In this way, they were isolated from each other, since they were then stranded at the former shoreline like detritus left high on a beach at a falling tide. The fauna found in anchialine habitats are frequently disjunct, relictual taxa (Ilfé 1990), thought to descend from shallow marine populations (Pérez-Moreno et al. 2016). This is a common pattern seen in many higher crustacean taxa known from anchialine systems, such as remipedes (Koenemann et al. 2009). This idea implies vicariance by continental drift (Bauzà-Ribot et al. 2012; Humphreys and Danielopol 2006), an hypothesis which is still contentious for some taxa (Phillips et al. 2013).

Anchialine species are, by definition, connected with the ocean, and so, more than in other subterranean systems, there is a need to distinguish between vicariance (earth history events) (McCarthy 2011) and dispersal (biological events) in their historical biogeography (Pérez-Moreno et al. 2016). The occurrence of the core anchialine taxa on seamount islands has been addressed repeatedly and oceanic dispersal considered likely (Humphreys and Danielopol 2006), however improbable it may seem (de Queiroz 2014). *Halosbaena*, with four described species, is the most widespread thermosbaenacean genus, occurring through the full extent of the Tethyan area in the Caribbean (*H. acanthura* Stock, 1976: Curaçao, Colombia, Jamaica, Cuba), eastern Atlantic (*H. fortunata* Bowman and Ilfè, 1986: Lanzarote in the Canary Islands), Indian Ocean (*H. tulki* Poore and Humphreys, 1992: north-western Australia; and an undescribed species from Christmas Island; Humphreys 2014) and the Philippine Sea (*H. daitoensis* Shimomura and Fujita, 2009, Minamidaitōjima, Okinawa Prefecture in the Ryukyu Islands) (Fig. 1). The latter two locations are isolated seamounts rising from abyssal depths. Species of this genus occur in shallow marine sands, the marine interstitial zone and fully marine caves (Wagner 1994; Wilkens et al. 2009), and in hyporheic and parafluvial sites in freshwater and down deep boreholes (>50 m) in karst (this paper).

Because of the extreme distances between the various *Halosbaena* species, which occur on oceanic and continental islands, as well as continents, they can serve as a useful surrogate in understanding the processes that have led to the observed pattern of its possible Tethys-influenced distribution,

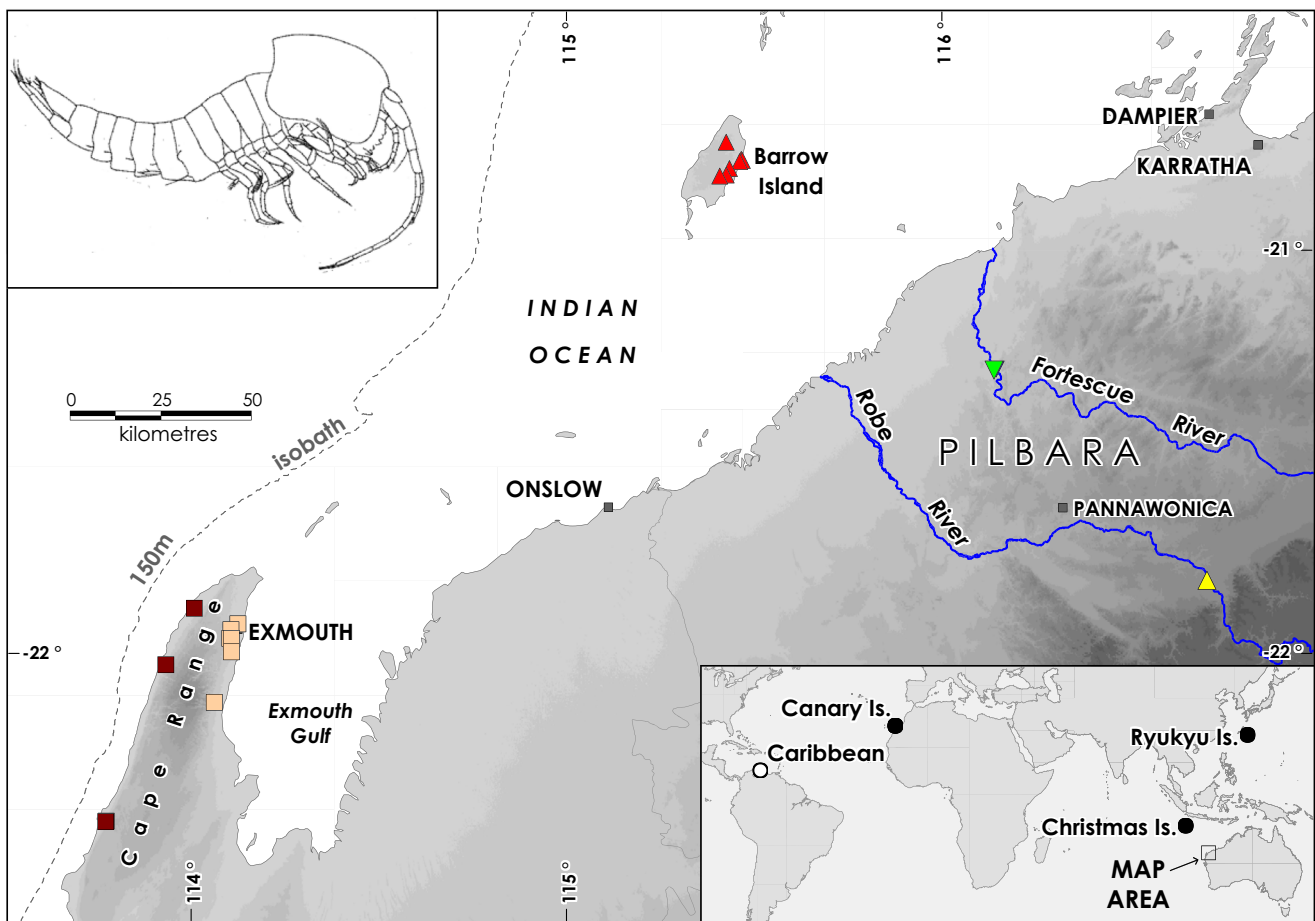


Fig. 1 Locations of *Halosbaena* species worldwide (bottom-right inset) and in north-western Australia (main map). Putative Australian species: squares W. side Cape Range *H. tulki*, squares E. side Cape Range *H. sp.*

CRE, triangles Barrow Is. *H. sp.* BI, inverted triangle Fortescue R. *H. sp.* PL, triangles Robe R. *H. sp.* PL. Top-left inset: *Halosbaena tulki* from Poore and Humphreys (1992)

but only if the systematic relationships between the different species can be unravelled and ideally located in geological time (Wagner 1994). There have been few detailed, cladistic morphological studies of *Halosbaena* (but see Wagner 1994), and anyway this is extremely difficult for subterranean taxa,

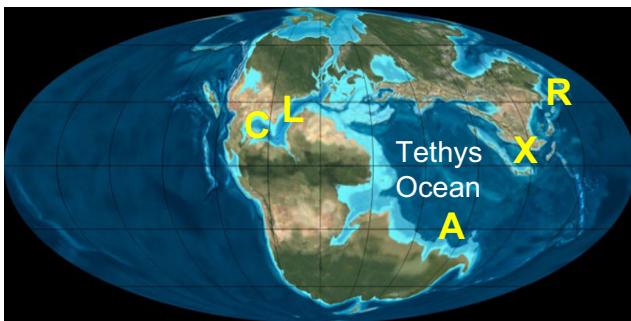


Fig. 2 Global palaeogeographic reconstruction of plate tectonics from the Middle Jurassic (170 million years ago) in Mollweide projection showing Tethys Ocean (map copyright: Ron Blakey, Colorado Plateau Geosystems, used with permission). Approximate locations of modern distribution: A Australia; C Caribbean; L Lanzarote; R Ryukyu Is.; X Christmas Is.

which frequently quickly converge on very similar morphologies to survive their challenging dark, energy-poor environment (Bishop and Iliffe 2012). Similarly, inferences of the timing of speciation are problematic as there are no fossils for this entire order (Jaume 2008). This requires the addition of molecular data for both phylogenetic and dating clock analyses (time-trees; Phillips et al. 2013), which are lacking for this order (Pérez-Moreno et al. 2016).

A detailed framework of the relationships of *Halosbaena* species is needed to understand worldwide patterns, and could also help to answer other questions at smaller scales within the subterranean biogeography of the Southern Hemisphere. For example, do the recently discovered *Halosbaena* specimens on Christmas Island represent a new species, and if so how does it relate to other *Halosbaena* species from different landmasses? Within the continental Australian context, *Halosbaena tulki* is recognised as a single species whose distribution extends at least 310 km, and traverses mountain ranges, islands and elevations to 300 m in three highly discrete, karstic areas (Cape Range, Barrow Island, Pilbara; see Fig. 1), all acknowledged hotspots for subterranean

biodiversity (Guzik et al. 2010; Hose et al. 2014) which abound with short-range endemic species (Harvey et al. 2011). The subterranean decapod shrimp *Stygiocaris* Holthuis, 1960 is also found in these same three areas, and molecular work has shown contrasting patterns of the existence of cryptic species within and between areas, as well as single species legitimately shared across the region (Page et al. 2008; De Grave et al., unpublished data), thus it is unclear what the relationships may be among *H. tulki* populations. Stygobitic species (subterranean aquatic restricted) rarely have ranges of greater than 200 km, and frequently much smaller (Trontelj et al. 2009), as found in a recent phylogeographic study of Mediterranean thermosbaenaceans from another family (Cánovas et al. 2016).

Within the Pilbara region itself, there is the unusual situation of an “anchialine” species being found at the Robe River at 300 m elevation in full freshwater over 100 km inland. If the exception proves the rule, this could have resulted from the stranding of a shallow coastal marine species in the Eocene when the coastline was much further inland via a marine transgression (Humphreys 2001a).

There is a further geographic question at an even smaller scale, this one on the Cape Range peninsula. On the western side is an apparently continuous, coastal plain, where there are populations of *H. tulki* and other sympatric stygofauna species. Previous work has shown genetic breaks between populations of the blind shrimp *Stygiocaris* (Page et al. 2008) and the blind fish *Milyeringa* Whitley, 1945 (Larson et al. 2013), suggesting subtle barriers to dispersal may also exist for *H. tulki*.

Here, we address a number of biogeographic questions relating to the relationship between landscape and evolutionary history at diminishing geographic and systematic scales within the thermosbaenacean genus *Halosbaena* using a combination of molecular phylogenetic tree hypotheses and time-tree analyses, namely, (1) the global relationships of *Halosbaena* species and the influence of the Tethys on the same, (2) the phylogenetic placement of Christmas Island *Halosbaena*, (3) whether *H. tulki* is a single, widely dispersed, species, (4) whether the possible stranding of ancestral *Halosbaena* in Pilbara uplands of Western Australia in the Eocene makes sense in light of our data, and (5) whether the phylogeographic breaks evident in other sympatric subterranean species in the western plain of the Cape Range, Western Australia, also exist within *H. tulki*.

Materials and Methods

Specimen collection

The genus *Halosbaena* occurs in continental Australia on land bordering the North West Shelf (see 150-m isobath in Fig. 1).

Field sampling covered the entire reported range of the only described Australia species, *H. tulki* (Fig. 1), with 13 sites on the Cape Range peninsula (Knott 1993; Poore and Humphreys 1992), 7 sites on Barrow Island (Humphreys et al. 2013; Humphreys 2001c) and 3 sites in the Pilbara (Eberhard et al. 2005; Halse et al. 2014) (see Table 1; Online Resource Table S1). Sampling took place between 2007 and 2012. A new, undescribed species of *Halosbaena* was also collected in 2010 from Christmas Island, a small, isolated limestone seamount near Java, approximately 1600 km north-west of the Pilbara coast in the Indian Ocean (Humphreys 2014). We also included specimens of other *Halosbaena* species from across its worldwide distribution (Fig. 1), including *H. daitoensis* from Minamidaitōjima in Okinawa in the Ryukyu Islands in the Philippine Sea (Shimomura and Fujita 2009) and *H. fortunata* from Lanzarote in the Canary Islands in the eastern Atlantic Ocean off the north-west coast of Africa (Bowman and Iliffe 1986). The only species of the genus from which we were unable to source a specimen is *H. acanthura* from the Caribbean Sea area (Stock 1976), despite extensive efforts by the last author in the field and via colleagues.

Sampling was achieved in three ways. Firstly, the parafluvial gravels of the Pilbara sites were sampled using a Bou-Rouch pump. This pumps water through a hollow spike driven into the alluvium and passes the effluent through a net (Fig. 3a) (Bou and Rouch 1967). Secondly, the caves of Christmas Island were sampled by hand net or directly into a vial by hand (Fig. 3b). Thirdly, the groundwater bores of Barrow Island and the Cape Range, which had originally been drilled for investigation, supply or monitoring, were sampled using small weighted plankton nets (mesh 250 or 350 µm) drawn up and down through the water column (tea bagging) up to 69 m below the surface. Samples were sorted in the field and fixed in 100 % ethanol and refrigerated. The most intact specimens from each site (with the same corresponding museum collection numbers; see Table 1) were retained separately for a complementary taxonomic and morphological study (King et al., unpublished data). Collecting was conducted under permit (Christmas Island: fauna AU-COM2013-181; Regulation 9.03, PS2013/015, Department of Sustainability, Environment, Water, Population and Communities; Western Australia: fauna, SF006957, land access CE002460, Department of Environment and Conservation). Colleagues kindly provided specimens from the Ryukyu Islands and Canary Islands (see Acknowledgments).

Laboratory

DNA was extracted from whole individuals using CTAB/phenol–chloroform (Doyle and Doyle 1987). Four different gene regions were sequenced (one mitochondrial, three nuclear) so as to target different systematic levels. Mitochondrial cytochrome *c* oxidase subunit I (COI) was

Table 1 *Halosbaena* specimens sequenced for this study

Area	Site	Taxon	DNA sequence no.				Latitude	Longitude	WAM collection no.
			COI	H3	28S	18S			
Australia	Barrow Is.								
	X62m	<i>H. sp. BI</i>	4	2	4	−20.7328	115.4258	16273	
	MW34	<i>H. sp. BI</i>	1			−20.7753	115.4661	16253	
	S4	<i>H. sp. BI</i>	1	1	1	−20.7781	115.4692	16261	
	GW05	<i>H. sp. BI</i>	4	1	2	−20.7808	115.4617	16251	
	S5	<i>H. sp. BI</i>	2		1	−20.7981	115.4347	16267	
	S8	<i>H. sp. BI</i>	7	1	2	−20.8131	115.4258	16262	
	M52	<i>H. sp. BI</i>	1			−20.8161	115.4081	16271	
	Cape Range								
	East - C-27	<i>H. sp. CRE</i>		1	1	−21.9272	114.1250	11194	
	East - WC17	<i>H. sp. CRE</i>		1		−21.9415	114.1053	16538	
	East - WC30	<i>H. sp. CRE</i>		1		−21.9626	114.1030	16542	
	East - WC29	<i>H. sp. CRE</i>		2		−21.9633	114.1073	16537, 16545	
	East - DS0 2/96	<i>H. sp. CRE</i>		1		−21.9958	114.1074	16536	
	East - Kailis 94	<i>H. sp. CRE</i>	1	3	3	−22.1216	114.0606	11199, 16113, 17202	
	West - C-25	<i>H. tulki</i>	2	2	1	−21.8883	114.0089	9782	
	West - C-215	<i>H. tulki</i>	13	2	3	−22.0278	113.9319	16117, 16118	
	West - MB	<i>H. tulki</i>	1	1		−22.4157	113.7732	17413	
	West - South Yardie	<i>H. tulki</i>		2	1	−22.4170	113.7718	14811	
	West - MW 149	<i>H. tulki</i>	1	1		−22.4171	113.7721	15040	
	Pilbara								
	Low - Fortescue River 1	<i>H. sp. PL</i>	3	1	2	−21.2954	116.1444	16176	
	Low - Fortescue River 2	<i>H. sp. PL</i>	5	1	2	−21.2978	116.1397	16173	
	High - Upper Robe River	<i>H. sp. PH</i>	2	3	4	−21.8186	116.7070	16181, 16182	
	Christmas Is.	Whip Cave (CI-54)	<i>H. sp. CI</i>	1	1	1	−10.4230	105.7013	18000
	Ryukyu Is.	Minamidaitōjima, Okinawa	<i>H. daitoensis</i>	1	1	1	25.8200	131.2200	16843
	Canary Is.	Túnel de la Atlántida, Lanzarote	<i>H. fortunata</i>	1	1	1	29.1500	−13.4200	17201
		Totals		49	30	30			5

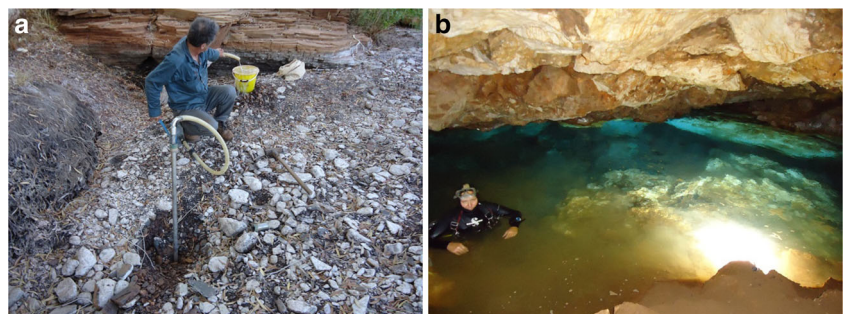
WAM Western Australian Museum

chosen because of its demonstrated ability to differentiate species and even populations within species (Page et al. 2008). Nuclear Histone (H3) was also sequenced and, while it is more conserved than COI, it can still distinguish between closely related species (Ashelby et al. 2012). Two nuclear ribosomal DNA regions were also sequenced (28S rDNA, 18S rDNA) to

provide deeper resolution of the species tree and allow comparison with the very few published sequences available within the order Thermosbaenacea for molecular dating.

The following primer sets were used to amplify a fragment of each gene using the polymerase chain reaction; COI: LCO-1490 and HCO-2198 (Folmer et al. 1994); Histone: H3-F and

Fig. 3 Sampling for *Halosbaena*: **a**) the Upper Robe River, Pilbara (photo: Rae Young; used with permission); **b**) Whip Cave, Christmas Island (Photo: Bill Humphreys)



H3-R (Colgan et al. 1998); 28S rDNA: 1274 and 1275 (Stenderup et al. 2006); 18S rDNA: 18Sai and 18Sb3.0 (Whiting et al. 1997) (see Table S2 for full primer/PCR details). Sequences were produced on an ABI 3130xl capillary auto-sequencer at Griffith University using the BigDye Terminator v.1.1 Cycle sequencing kit and edited using Sequencher 4.1.2 (Gene Codes Corporation).

Phylogenetic analyses

Each of the four single gene datasets was separately imported into Mega6 (Tamura et al. 2013) and aligned using Muscle (Edgar 2004) with default settings within Mega. The sequences were translated into amino acids for the two protein coding genes (COI: Invertebrate Mitochondrial Genetic Code; H3: Standard Genetic Code) to look for stop codons and anomalous codons, indicative of non-functional nuclear paralogues. The most appropriate substitution model (lowest Bayesian Information Criterion score) was selected for each gene with Mega.

Ten separate datasets were created from the resulting sequences for subsequent analyses at different systematic and biogeographic levels of interest (Table 2). These included four single gene datasets (COI, H3, 28S, 18S), four combined datasets of two genes (COI/H3, COI/28S, H3/28S, 18S/28S), two datasets of three genes (COI/H3/28S, H3/28S/18S) and one dataset of all four genes together (see “Molecular Dating Analyses” for more information on the

final dataset). The two- and three-gene datasets only included individuals that had been sequenced for all the respective loci and so include a reduced selection of individuals from the relevant single gene datasets; thus, further model searches were run for each reduced single gene portion within Mega to select the appropriate model.

Two forms of phylogenetic analysis were used for the single gene datasets with the relevant substitution model, Bayesian Analysis in MrBayes v.3.2.2 (Ronquist et al. 2012) and Maximum Likelihood in Mega. Parameters used were: Bayesian Analysis: 1–2 million generations (until stationarity reached) trees sampled every 1000 cycles, 25 % burn in, and two runs of four chains heated to 0.2; Maximum Likelihood: 500 bootstrap replicates. Bayesian analysis in MrBayes was used for the various multi-gene datasets as above but with each dataset partitioned by gene with relevant substitution model for each gene. The MrBayes and Mega trees were mid-point-rooted except for the 18S/28S tree, which was rooted with the outgroup *Tethysbaena argentarii* (Stella, 1951) (see next section). FigTree v.1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>) was used to annotate all trees.

Haplotype networks were constructed for selected Australian clades where complete homologous alignments were achieved (i.e. no missing data; see Joly et al. 2007) to explore smaller scale phylogeographic relationships between haplotypes in TCS v.1.21 (Clement et al. 2000) using statistical parsimony. Genetic distances between highly divergent monophyletic clades were calculated in Mega (net between-

Table 2 Different datasets, molecular models and tree scores for phylogenetic analyses conducted in this study

Loci/Dataset	No. indiv.	Geographical Areas included ^a	Program	Model ^b	Tree scores		Figure
					Bayesian	Likelihood	
COI	49	BI, CR, PIL, CI	MrBayes/Mega	HKY+G+I	-2544.94	-2407.03	S1, S5
H3	30	BI, CR, PIL, CI, MJ, LZ	MrBayes/Mega	K2+G	-1204.81	-1118.61	S2, S6
28S	30	BI, CR, PIL, CI, MJ, LZ	MrBayes/Mega	T92+G	-835.13	-552.70	S3, S7
18S	5	BI, CR, CI, MJ, LZ	MrBayes/Mega	K2	-661.69	-650.91	S4, S8
COI/H3	10	BI, CR, PIL, CI	MrBayes	As per gene	-3095.39		S9
COI/28S	9	BI, CR, PIL, CI	MrBayes	As per gene	-2708.16		S10
H3/28S	12	BI, CR, PIL, CI, MJ, LZ	MrBayes	As per gene	-1852.67		S11
18S/28S	6	BI, CR, CI, MJ, LZ, IT	MrBayes	K2+I	-1856.10		S12
COI/H3/28S	9	BI, CR, PIL, CI	MrBayes	As per gene	-3551.32		S13
H3/28S/18S	5	BI, CR, CI, MJ, LZ	MrBayes	As per gene	-2345.80		S14
COI/H3/28S/18S	12	BI, CR, PIL, CI, MJ, LZ, IT	BEAST	As per gene	-5202.55		4
COI	17	CR-W	TCS	Parsimony			5
COI	20	BI	TCS	Parsimony			S15
H3	17	CR	TCS	Parsimony			S16
28S	27	BI, CR, PIL	TCS	Parsimony			S17

^a BI Barrow Island; CR Cape Range; CR-W Cape Range West; PIL Pilbara; CI Christmas Island; MJ Minamidaitōjima (Ryukyu Is.); LZ Lanzarote (Canary Is.); IT Italy

^b G Gamma Rate Distribution; HKY Hasegawa-Kishino-Yano; I invariant sites; K2 Kimura 2-parameter; T92 Tamura 3-parameter

group mean distances) using the Kimura 2-parameter (K2P) model for each gene.

All input datasets for all analyses are available online at the Dryad Digital Repository (doi:10.5061/dryad.nh7pg).

Molecular dating analyses

A four-gene dataset was created from which to derive molecular dating estimates (time-trees) using BEAST 2 v.2.1.3 (Bouckaert et al. 2014). Exemplars from all the areas and lineages identified in the analyses above were included, and 28S and 18S sequences of *Tethysbaena argentarii* were downloaded from GenBank and used as an outgroup for rooting the trees (accession numbers DQ470654, AY781415) (Spears et al. 2005; Stenderup et al. 2006) (Table S1). Data were coded as missing for individuals that had not been sequenced for all four genes as recent work has found exclusion of some specimens with missing data could decrease accuracy (Jiang et al. 2014). Each of the different loci used the relevant molecular model for each locus as implemented in BEAUti 2 (part of the BEAST package). We used an uncorrelated lognormal relaxed-clock (unless specified) and assumed a Yule model of speciation. Each analysis entailed 100 million MCMC generations with parameters sampled every 100 generations (trees every 1000 generations). We used a burn-in of 10 % for all parameters using Tracer v.1.5 (part of BEAST) and sought convergence by ensuring effective sample size (ESS) values were greater than 200 for all node height, prior, posterior and likelihood parameters. We used TreeAnnotator v.2.1.2 (part of BEAST) with a burn-in of 10 % to derive maximum clade credibility trees with mean node heights and trees were visualised in FigTree.

We ran a total of nine different BEAST time-tree analyses. For the first analysis, we did not impose any topological constraints or calibration bounds so as to compare the topology derived from BEAST with those obtained from MrBayes and Mega, and to get support values for all nodes. Guided by the MrBayes, Mega and preliminary BEAST results, we then enforced monophyly on the following areas/taxa; (1) all *Halosbaena*; (2) Ryukyu Islands and Canary Islands; (3) continental Australia and Christmas Island; (4) continental Australia; (5) Barrow Island; (6) Cape Range; and (7) Cape Range West.

For the second analysis, we used a strict molecular clock by we imposing a substitution rate of 0.0125 per million years (Ketmaier et al. 2003), which is a published rate from subterranean isopods similar to most general invertebrate COI rates (Wilke et al. 2009), on the COI portion of the dataset and allowed the other loci to be estimated relative to COI, and then estimated the ages of various nodes. For the remaining seven analyses, we did not employ any substitution rate, but instead used an uncorrelated lognormal relaxed-clock model and imposed prior calibration bounds on a number of nodes and

enforced monophyly as above. We sought calibrations from multiple topological depths (Wheat and Wahlberg 2013) and from both sides of the deepest *Halosbaena* node so that dates are interpolated between calibrations (Thorne and Kishino 2002). As therosbaenaceans do not have a known fossil record (Jaume 2008), we used a combination of calibration bounds derived from biogeographical and geological information as an independent way of bringing the *Halosbaena* phylogeny into actual years ago rather than just relative ages (Duchêne et al. 2014). Although there are geological age estimates for a number of the islands on which species of *Halosbaena* are found, these are inadequate for calibration since the age of a lineage may bear no relation to the age of an island. This is because colonisation may have occurred well after the formation of the island, or indeed beforehand, as there are older, submerged seamounts in many archipelagos (Heads 2011), and therefore simple island ages cannot properly constrain a node. Instead, we sought biogeographic events that may have sundered existing populations or potential dispersal routes, and thus created a barrier to gene flow and allowing subsequent divergence.

We identified three nodes around which to impose calibrations bounds:

A) The youngest of these is the common ancestor of the two sister taxa found on opposite sides of the Cape Range (western: *H. tulki*; eastern: *H. sp.* CRE), and thus presumably sundered by the uplift of this orogeneic zone, as has been hypothesised for other Cape Range stygobitic species with similar distributions (Page et al. 2008). Uplift of the Cape Range was completed no later than the late Pliocene (2.6 MYA) and may date from Mid-Miocene (11.2 MYA) (Wyrwoll et al. 1993), and so we defined a uniform distribution with hard constraints for this node of 11.2–2.6 MYA as we have no prior information about precisely when this event may have happened within this range of dates.

B) The second calibration node is that joining the far-flung sister taxa from the Ryukyu and Canary Islands (*H. daitoensis* and *H. fortunata*). The ancestor of this clade may well have inhabited the intervening Tethys Sea, as it is extremely hard to construct a scenario for their common ancestor that does not in some way include a Tethyan distribution or dispersal of some description. Thus the final closure of the Tethys Seaway around 10 MYA is likely the latest possible date when this speciation event could have occurred (McQuarrie et al. 2003). We have defined a uniform distribution for this node of 25–10 MYA with hard constraints.

C) The oldest calibrated node is that of the root, perhaps the most important node to calibrate (Duchêne et al. 2014). The root joins *Halosbaena* and *Tethysbaena*. Jaume (2008) and Cals and Monod (1988) suggest that this divergence may be related to the maximum extent of the Tethys and final opening of the Atlantic. As the timing of the opening of the Atlantic is fairly elastic, we have taken the presence of a continuous band

of ocean crust through the length of the North Atlantic to represent the continuous ocean basin, which coincides with the J-anomaly, the Jurassic-Cretaceous boundary at 118 MYA (Vogt and Tucholke 1989). Given the lack of precision around these dates, we have defined a normal distribution around this node centred on 107.5 MYA with 95 % of the range encompassing 125–90 MYA with soft constraints to reflect the bidirectional uncertainty of this node (Wheat and Wahlberg 2013).

Imposing any kind of calibration bound is fraught with difficulties as one can never be sure if the event invoked truly influenced the node with which it has been associated. To test the influence of each one of the three calibration bounds on our results, we initially ran our time-tree analyses only using one of the calibration bounds at a time, and then ran analyses using each combination of two calibrated nodes. We could then infer the ages of each of the calibrated nodes from analyses in which they had not been included as calibrations, and thus compare the inferred ages to the bounds that we have chosen for them to see if they were similar. This was also to see if the three calibration bounds were internally consistent with each other or told conflicting stories, and whether one bound had an undue influence on the results.

Results

Sequences and molecular models

We generated 120 new sequences from 70 specimens, of which 55 were COI [557 base pairs (bp), GenBank accession numbers KT984044–KT984092], 30 H3 (328 bp, KT984093–KT984122), 30 28S (303 bp aligned, KT984014–KT984043), and five 18S (332 bp aligned, KT984009–KT984013) (see Table 1; Online Resource Table S1 for all details). We removed six of the Pilbara COI sequences as being probable pseudogenes because, even though the chromatograms were clear and crisp, they showed an obvious single nucleotide deletion leading to a frameshift, as well as being suspiciously divergent (Buhay 2009), a pattern not seen in COI sequences from other individuals from the same site nor from other loci sequenced from the same individuals with the suspected COI pseudogenes.

COI sequences were recovered from all three continental Australian areas (Barrow Island, Cape Range, Pilbara) and Christmas Island, but not from the Canary or Ryukyu Islands. H3 and 28S sequences were recovered from all areas. For 18S, only exemplar specimens were sequenced, which included two individuals from continental Australia (Barrow Island, Cape Range), as well as one each from Christmas Island, the Canary Islands and Ryukyu Islands.

Nucleotide substitution models were selected for each single gene dataset (Table 2). Models selected for the reduced

datasets to be included in the various multi-gene analyses proved to be the same model as for the relevant full single gene dataset.

Phylogenetic analyses and clades

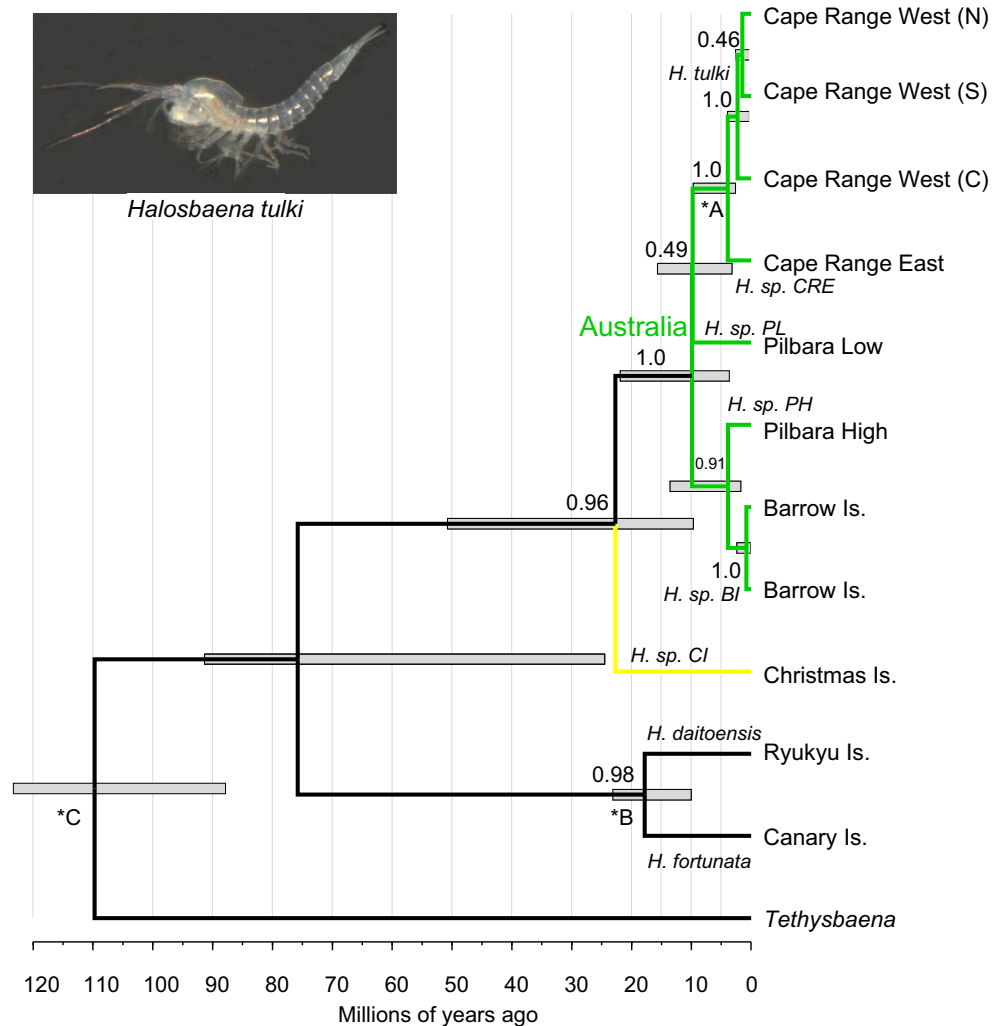
Various combinations of single and multi-gene datasets were analysed (Table 2; Figs. 4, 5; Online Resource Figs S1–S17), with biogeographic and systematic implications at various levels. The topology from the BEAST analysis that included no topological constraints or calibration bounds (support values from Fig. 4) generally agree with the topologies from the one-, two- and three- gene analyses done in MrBayes and Mega (Figs S1–S14).

At the largest geographic and systematic scale is the relationship between the various *Halosbaena* taxa spread liberally across the globe. There appears to be a basal split between taxa from the Northern Hemisphere and those from the South. Northern taxa, *Halosbaena daitoensis* (from Ryukyu Islands) and *H. fortunata* (from Canary Islands), are strongly recovered as sister species in all relevant analyses with strong support (>0.98), as are Southern Hemisphere taxa from continental Australia and Christmas Island (>0.96) (Fig. 4). However the divergence between Ryukyu Islands and Canary Islands (9.50 % at H3, 0.87 % at 28S, and 1.85 % at 18S) is considerably less than that between Christmas Island and Australian taxa. However, when the *Halosbaena* sequences were rooted with *Tethysbaena* (Figs. 4, S12), the Christmas Island taxon groups strongly with Australia, thus forming a Southern Hemisphere clade.

Given the high level of divergence between the newly discovered Christmas Island species and all other species, it appears to be a separate lineage and is likely to be a new species. The Christmas Island species (hereafter informally called *Halosbaena* sp. CI) is 20.90 % divergent at the mitochondrial COI locus (K2P model) from its Australian sister taxa, and at nuclear loci is 17.99 % at H3, 7.68 % at 28S, and 3.73 % at 18S.

The continental Australian specimens from three areas (Cape Range, Barrow Island, Pilbara) appear to form a strongly supported single phylogenetic lineage, but likely constitute a number of very distinct taxa, some of which are probably separate species (Fig. 4). Although the exact number of Australian *Halosbaena* species represented here will require further detailed systematic and taxonomic work, provisionally there seems to be five distinct putative species that clearly reflect geography: (1) Barrow Island (*Halosbaena* sp. BI); (2) Cape Range – eastern side (*H.* sp. CRE); (3) Cape Range – western side (encompasses the type location, so is likely true *Halosbaena tulki*) (Poore and Humphreys 1992); (4) Pilbara – high elevation (*H.* sp. PH); and (5) Pilbara – low elevation (*H.* sp. PL). Molecular distances between the five taxa range from 16.42 to 20.79 % for COI and 1.27–5.85 % for H3.

Fig. 4 *Halosbaena* time-tree using four loci (COI, H3, 28S, 18S) and three biogeographic calibrations: *A Cape Range uplift (11.2–2.6 MYA, uniform distribution, hard constraints); *B closure of Tethys Seaway (25–10 MYA, uniform distribution, hard constraints); *C: opening of Atlantic and maximum extent of the Tethys (normal distribution centred on 107.5 MYA, 95 % 125–90 MYA, soft constraints). Posterior probability support values from topological/calibration free analysis. *Inset* *Halosbaena tulki* (Western Australian Museum)



Among the five divergent continental Australian lineages (*H. sp. BI*, *H. sp. CRE*, *H. tulki*, *H. sp. PH*, and *H. sp. PL*), the strongest and most obvious sister relationship is between Cape Range East (*H. sp. CRE*) and Cape Range West (*H. tulki*). While certainly distinct from each other (14.46 % COI, 1.24 % H3; Figs S1, S2, S16), they strongly group together in most of the analyses, including the combined four-gene analysis (Fig. 4). However, the relationships among the other continental species are less certain and will require more data, although there are some suggestive patterns.

Pilbara Low (*H. sp. PL*) and Pilbara High (*H. sp. PH*) are clearly different species as they do not form any sister relationships with each other and are highly divergent (14.34 % COI, 4.40 % H3), unsurprising given the sites are 80 km apart at different elevations. *Halosbaena sp. PH* may however form a clade with *H. sp. BI*, as this relationship occurs in a number of the analyses including in the combined four-gene one (posterior of 0.91); however, the precise delineation and relationship of the continental Australian species will require more data.

Two of the putative species displayed intraspecific phylogeographic patterns. Within *Halosbaena sp. BI* from Barrow Island, there are three distinct populations visible within the COI data, which correspond to sites GW05 and X62m, and to five sites for the third group (1–3.64 % COI) (Fig. S15). Within *H. tulki* on the western side of the Cape Range, there are three clear phylogeographic groupings that equate to the north (site C-25), central (site C-215), and south (sites MB, MW 149, South Yardie) of the western Cape Range peninsula (COI 5–6 % divergent) (Fig. 5).

Molecular dating estimates

Effective sample size (ESS) values for all analyses were greater than 226 for the posterior and prior statistics, greater than 2409 for the likelihood statistic, and greater than 1723 for all MRCA times for nodes of interest, suggesting good mixing and an effective MCMC sampling of the posterior distribution.

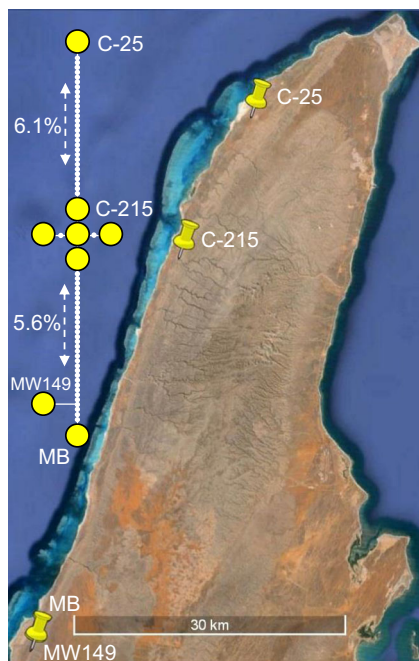


Fig. 5 COI haplotype network of *Halosbaena tulki* from the western side of the Cape Range peninsula related to their sampling sites (map: Google Earth)

We estimated ages for four nodes that were not associated with any calibrations (Table 3). The youngest uncalibrated node of interest was the most recent common ancestor of the three *H. tulki* lineages in the western Cape Range, which returned a mean estimate at 1.66 MYA with 95 % highest posterior density (HPD) of 3.92–0.28 MYA in the analysis which included all three calibrations (A, B, C) (Table 3; Fig. 4). The lowest mean estimates were all in analyses that only included a single calibration, and the highest in the Strict COI analysis (Table 3). The next uncalibrated node mean estimate is that which joins all the continental Australian

lineages, with a mean value of 11.09 MYA (22.05–3.71) in the 3 calibration analysis. The lowest mean estimates are the three analyses with only a single calibration and the Strict COI analysis (3rd lowest), whereas the highest mean estimate was in the two calibration analysis which used only the two older calibrations (B, C) (Table 3). The mean age of the node linking Australia and Christmas Island was dated to 27.99 MYA (51.61–10.22) in the 3 calibration analysis, with the lowest estimates derived from the single calibration analyses and the Strict COI analysis (2nd lowest), and the highest from one of the two calibration analyses (B, C). The mean MRCA for *Halosbaena* species was 52.86 MYA (90.98–23.43) in the 3 calibration analysis, with the youngest estimate in the Strict COI analysis, and next youngest in the single calibration analyses that used the younger calibrations (A, B), with the oldest mean estimate from the two calibration analysis (B, C).

The age of each one of the three calibrated nodes was estimated many times in the numerous permutations of the one- and two-calibration analyses that did not include a constraint for the relevant node of interest (A: Cape Range Uplift / *H. tulki* and *H. sp. CRE*; B: closure of Tethys Seaway / *H. daitoensis* and *H. fortunata*; C: opening of Atlantic / *Halosbaena* and *Tethysbaena*; see “Materials and methods”) (Table 3). When the MRCA of *H. tulki* and *H. sp. CRE* was estimated without the Cape Range calibration (A), the means ranged from 5.72 to 4.43 MYA (13.38–0.70), which agrees reasonably well with the hard calibration bounds imposed in other analyses (upper 11.20 MYA, lower 2.60, mean 6.90). When the Tethys calibration (B) was not included, the mean estimates for *H. daitoensis* and *H. fortunata* were 13.23–6.20 MYA (33.12–1.76) (relevant hard calibration bounds; upper 25.00, lower 10.00, mean 17.50). The estimates for the root node (*Halosbaena* and *Tethysbaena*) when the Atlantic calibration (C) was not used were 90.34–59.02 MYA (244.94–

Table 3 Estimated mean ages of most recent common ancestors from time-tree analyses (in millions of years ago)

Calibrations used ^a	CR west (<i>H. tulki</i>)	CR - east & west	Australia	Australia/ CI	<i>H. daitoensis</i> / <i>H. fortunata</i>	<i>Halosbaena</i> spp.	<i>Tethysbaena</i> / <i>Halosbaena</i>
Strict COI rate of 0.0125/MY	2.16	5.35	9.00	18.15	6.20	28.85	69.81
A	1.26	4.22 ^b	7.62	16.75	8.49	29.69	59.02
B	1.45	4.52 ^b	9.02	21.09	14.29 ^b	38.66	77.31
C	1.34	4.43	8.78	22.77	11.22	42.18	105.20 ^b
A, B	1.60	5.01 ^b	9.88	23.53	14.82 ^b	42.89	90.34
A, C	1.54	5.05 ^b	10.28	26.47	13.23	49.79	105.84 ^b
B, C	1.78	5.72	11.45	28.11	15.10 ^b	52.68	106.08 ^b
A, B, C	1.66	5.36 ^b	11.09	27.99	15.27 ^b	52.86	106.15 ^b

Abbreviations: CI Christmas Island, COI cytochrome *c* oxidase subunit I, CR Cape Range, MRCA most recent common ancestor, MY million years

^a Calibrations: A Cape Range Uplift; B closure of Tethys Seaway; C opening of Atlantic

^b MRCA of node subjected to calibration bounds

5.27) (soft calibration bounds; upper 125.00, lower 90.00, mean 107.50).

On the whole, the three calibration bounds do not conflict with each other as the estimates of the ages of each calibrated node falls within the range assumed for bounds; however, the fit is not perfect. When the two uniform calibration bounds (A, B) were used, their posterior distributions were often tightly pressed against their minimum bounds, and the estimates for normally calibrated bound (C) were generally lower than the imposed range of bounds. There is a visible effect of the influence of different calibrations, with analyses employing only a single calibration returning generally younger age estimates, the strict COI rate analysis returning older estimates for shallow nodes and younger estimates for deeper nodes (probably reflecting saturation) (Wheat and Wahlberg 2013), and the two calibration analysis (B, C) that left out the youngest calibration (A) returning older ages. Shallow calibrations on their own can compress the tree and lead to younger estimates for deep nodes, and deep calibrations can extend the branches leading to older estimates for shallow nodes (Phillips 2009). However, the age estimates were generally internally consistent with each other (Table 3), but that does not mean that they are necessarily correct. But it does mean that, if they are wrong, then at least they are all wrong in the same manner and to the same degree, and so can be used together in the same analysis.

Discussion

Global *Halosbaena* relationships and the Tethys

Rooted phylogenetic analyses agree on a deep divergence between taxa from the Northern (Canary and Ryukyu Islands) and Southern Hemisphere (Christmas Island and Australia). The relationship between Australia and Christmas Island may not appear especially surprising given their relative proximity to each other (1600 km) and the sharing of various subterranean genera between them (Humphreys 2014). However, the even stronger and closer relationship between the Canary and Ryukyu Islands is particularly interesting and potentially revealing, given the great distance between them (13,000 km). The one missing taxon in this analysis, *H. acanthura* from the Caribbean, could potentially have provided some insight to these relationships. Poore and Humphreys (1992) suggested that *H. acanthura* shared some features with *H. tulki* from Australia, which if reflecting a close relationship, would break the north/south divide. However, morphological cladistic analyses (Wagner 1994) recovered *H. acanthura* as sister to *H. fortunata* from Canary Islands, which instead implies a geographically more feasible Northern Hemisphere ampho-Atlantic relationship (Fig. 2).

Neither the evolutionary patterns of relationships of *Halosbaena* species inferred above nor the more general distribution patterns of anchialine faunas necessarily argue for the Tethyan hypothesis, which advocates larger-scale geological dispersal by tectonic plate movement (Humphreys 2008; Wilson 2008) and smaller-scale biological dispersal along the shores of land bordering the Tethys. Simple broad pattern matching is not really adequate for this; however, the addition of time estimates for the relevant biological and geological events may make the role of the Tethys clearer on the distribution and origin of *Halosbaena* (Wagner 1994).

Thermosbaenaceans only occur in the tethyan area, with the genus *Halosbaena* by far the most widespread example. Northern (Canary and Ryukyu Islands – Atlantic and Pacific oceans) and southern clades (Christmas Island and Australia – Indian Ocean) of *Halosbaena* may have diverged between the Late Cretaceous–Oligocene (Fig. 4), by which time Australia was well separated from Eastern Gondwana, and the Atlantic had opened up (Scotese 2015). As Australia was never close to the Atlantic region (Poore and Humphreys 1992), there would be no imprint on the distribution of *Halosbaena* species by changes in the Tethys coastline (Wilkens et al. 2009). The occurrence of species of *Halosbaena* on Christmas Island and the Ryukyu Islands, both isolated seamounts situated on the ocean-side of trenches and rising from abyssal depths, supports the scenario of an ancestor dispersing across the open ocean.

So, while the thermosbaenaceans are associated with the extent of Tethys, it does not seem terribly likely that the distribution and divergence of *Halosbaena* species is a direct result of the Tethys spread because the age of divergences are considerably too young (Fig. 4), they occur on seamounts, and the close relationship between the Ryukyu and Canary Islands makes clear that their ancestors were not passive passengers on shallow continental shelves being pushed around by ancient tectonic movements. Although the spreading Tethys appears unlikely to be the mechanism behind divergences seen within *Halosbaena*, the Tethys is almost certainly the medium by which their ancestors came to be where they ended up. It seems likely that *Halosbaena* species had widespread marine ancestors in Tethys with a preference for shallow marine caves and/or crevicular systems, and colonised different places at different times, not directly related to the spread of Tethys.

In fact, some have argued that *Halosbaena* is only a recent, even Pleistocene, invader of groundwater, given their marine proclivity, “primitive” ancestor-like appearance (Monod and Cals 1988), salinity tolerance (Stock 1976), wide distribution (Wagner 1990) and occurrence in fairly young geological environments (Bowman and Iliffe 1986; Stock 1976). This is difficult to prove as some features of *H. acanthura* are actually fairly derived (Stock 1976), and although karst environments may be young, there are often older sediments close by

(Wilkens et al. 2009). Recent work has reconstructed climates from Cape Range caves going back at least 27,000 years (Denniston et al. 2013); however, these same caves have other records going back at least half a million years (R. Denniston, personal communication). Anchialine habitat has probably existed in some form in the area for millions of years following the initial uplift (Wyrwoll et al. 1993), given the presence of flank-margin caves near the top of Cape Range (to 300 m), which indicates that suitable habitat has been present throughout the emergent stage in the orogeny of the range (Mylroie et al. 2015). This all means that estimating precisely when different *Halosbaena* taxa may have invaded the groundwater in each area is problematic and were likely independent events.

A number of other studies have used molecular data to infer the role of the Tethys in structuring subterranean crustacean populations, with varied results. For example, Bauzà-Ribot et al. (2012) and Hou et al. (2014) found some evidence for a Tethyan vicariant influence within their taxa of interest, whereas Botello et al. (2013), Page et al. (2008) and Phillips et al. (2013) found an inconsistency between geological and molecular dates, and Hoenemann et al. (2013) was inconclusive. The question of the precise relationship between the Tethys and the subterranean communities found on its edge would appear to lend itself to more formal, multi-taxon analyses to really understand how the process and patterns may relate.

As stated in “Materials and methods”, there are many potential issues with time-tree analyses, and any molecular dating has a raft of assumptions and sources of error (Santos and Weese 2011), making large errors possible (Trontelj 2007). Various studies have concluded that tectonically driven divergences have occurred between stygofauna on different continents (Bauzà-Ribot et al. 2012; Chakrabarty et al. 2012), while reanalyses have concluded precisely the opposite (De Bruyn et al. 2013; Phillips et al. 2013), highlighting the complex, amorphous nature of some of these questions. It is also possible that the addition of the Caribbean species could change the topology and timings with a new calibration point, although the strong support for the topology in most analyses would argue against this (Duchêne et al. 2014). Given that the Canary and Ryukyu Islands relationship is not likely due to the spreading of Tethys, it is possible that there could be an even closer trans-Pacific relationship between the Ryukyu Islands and the unsampled Caribbean species. These areas could have remained connected via gene flow until a relatively recent time with the formation of the barrier of isthmus of Panama between the Pacific and Caribbean ~3.5 MYA (perhaps with some other earlier episodes; Bacon et al. 2015). It is even possible that this putative relationship could itself be tectonically derived, since Curaçao is part of the Caribbean Plate that migrated through the region that is the “isthmus of Panama” from about the position of the

Galapagos from about the Late Cretaceous until the mid-Eocene (Pindell and Kennan 2009). And so, while this may appear a ridiculous geographic relationship, it is arguably less so than the one already demonstrated here between the Ryukyu and Canary Islands.

The problem may be with the very broad nature of the “Tethys” question itself, both geographically and temporally. The Tethys existed in one form or another for most of the last quarter of a billion years and extended halfway across the Earth (Fig. 2), so it is bound to have overlapped with a large number of divergences, both geological and biological. It may be a too large-grained “pixellation” of an idea and the scales too extensive to easily resolve many more fine-grained, specific questions. However, in the case of the genus *Halosbaena*, the Tethys appears to be the ancient stage upon which more contemporary events played out, rather than it being the prime engine for divergence.

Christmas Island *Halosbaena*

Given our data, there seems little doubt that the Christmas Island *Halosbaena* (*H.* sp. CI) is an undescribed species that is likely sister to mainland Australian taxa, and as such it will be formally described in a related study (King et al., unpublished data). But determining when and how it came to be there is another issue. The divergence between *Halosbaena* sp. CI and Australian taxa is estimated to have happened about 28 MYA, which does accord reasonably well with geological estimates for the age of the island (emergent at least 26 MYA, Grimes 2001; vulcanism 43–37 MYA, Taneja et al. 2015), although the subterranean habitat itself may be much younger (Humphreys 2014). However, even if the inferred divergence is accurate, it is not really possible to tie the divergence to a particular location, especially given that we have established that the genus *Halosbaena* has moved extensively during its history. It could be a previously vagile, ancient denizen of now submerged seamounts, or a recent immigrant, now detained at Christmas Island.

Halosbaena tulki species status

There is currently only a single, described species of *Halosbaena* in continental, north-western Australia, *H. tulki*. It was described in Poore and Humphreys (1992) from the western side of the Cape Range peninsula, but also reported later from the eastern side of the cape, and the Pilbara and Barrow Island areas separated by hundreds of kilometres. However, our data clearly indicate that this taxon includes a suite of unrecognised species (Figs. 4, S1–S14), which here we informally designate as *Halosbaena* sp. BI, sp. PH, sp. PL, and sp. CRE, with those individuals from the western side of the Cape Range presumably representing *H. tulki* sensu stricto due to it encompassing the type location. We will only know if

these “cryptic” species truly are distinct in a taxonomic sense following full morphological analyses backed up by more formalised multi-locus DNA species delineation methods (Fontaneto et al. 2015).

Subterranean species have often been reported to have surprisingly large distributions, especially water-living stygobionts, as the habitat is considered to be more connected than are the air-filled voids used by terrestrial troglobionts (Zakšek et al. 2009). However, molecular methods have shown that the presumed relatively large ranges of European stygobionts actually constitute distinct, cryptic lineages, and so true ranges of over 200 km for single species are exceedingly rare (Trontelj et al. 2009). Indeed, Cánovas et al. (2016) found cryptic species within the thermosbaenacean taxon *Tethysbaena scabra* (Pretus, 1991) with ranges no greater than 60 km. This is unsurprising given the often discontinuous nature of subterranean habitats, which, when coupled with time, is a recipe for divergence and speciation (Barr and Holsinger 1985). Cryptic species have been found using molecular phylogenetics in a number of Australian subterranean species (Page et al. 2007), including within the distributional range of *H. tulki* sensu lato. Tributaries of rivers in the Pilbara were found to contain genetically isolated populations of the stygobiont amphipods *Pilbarus* Bradbury & Williams, 1997 (Ashburton River) and *Chydaekata* Bradbury, 2000 (Fortescue River) (Finston et al. 2007). Similarly the atyid shrimp *Stygiocaris* shares the same three main areas as *H. tulki* sensu lato and hosts at least two cryptic species, on the western side of the Cape Range (Page et al. 2008) and the Pilbara coast (De Grave et al., unpublished data). The cave gudgeon *Milyeringa* has also recently been shown to have a cryptic species on Barrow Island (Larson et al. 2013), genetically distinct from populations at the Cape Range coast.

The suite of five putative *Halosbaena* species are all found in separate, distinct areas, and thus presumably their divergences have a geographical/geological explanation due to the many conspicuous and subtle landscape features. However, some of these are probably too recent to be phylogenetically consequential. For example, the ocean barrier separating Barrow Island, the Pilbara coast and Cape Range peninsula formed a continuous area of land as recently as the Holocene (Humphreys et al. 2013). Cape Range and Barrow Island are both anticlines of Tertiary carbonates, with the former probably elevated above sea level in the Late Miocene–Pliocene, reaching its current elevation by the Late Pliocene (Wyrwoll et al. 1993). For most of this time, a broad plain linked the Cape Range, Barrow Island and coastal Pilbara, which was subsequently inundated by the Holocene sea level rise, with the coast moving inland by as much as 125 km. This fragmented these previously connected areas (Fig. 1). This particular event is probably too recent to explain the extensive genetic divergence between the taxa, as is the delineation of the Robe and Fortescue river basins, because the Fortescue

captured the Robe in the Late Pleistocene–Holocene (Barnett and Commander 1985). However, other geographic features have been more prolonged barriers to dispersal and gene flow, such as the uplift of Cape Range itself in the late Tertiary (Wyrwoll et al. 1993) and elevated groundwater salinity at the base of the Cape Range peninsula (Allen 1987). The populations on the east side of Cape Range (sp. CRE) differ from those on the west, which includes the type locality for *H. tulki* (Fig. S16), and is likely due to the vicariant uplift of the Cape Range hills, a pattern also seen in the cave shrimp (Page et al. 2008) and cave gudgeons (Adams and Humphreys 1993).

Pilbara upland *Halosbaena*

In the Pilbara, *Halosbaena* sp. PL occurs in the low, coastal deltas of the Fortescue River, which is a typical environment for an anchialine species, but surprisingly another *Halosbaena* species (*H.* sp. PH) is also found 112 km inland at an elevation of 300 m in the Robe River, in the large Robe Palaeovalley (Fig. 1). Only three other thermosbaenaceans occur at similar elevations; *Monodella* Ruffo, 1949 (180 m; Maquire 1965), *Tethysbaena gaweini* Wagner, 1994 (up to 240 m) and *T. haitiensis* Wagner, 1994 (580 m; Wagner 1994). The best process to account for the presence of lineages with marine ancestry in isolated and elevated locations is the “two-step model” (Boutin and Coineau 1990), with an initial colonisation of marine interstitial spaces during periods of high sea-level, followed by stranding inland after sea-level retreat (Notenboom 1991; Stock 1980). Wagner (1994) suggested that the Australian thermosbaenaceans separated from the global distribution during the Mesozoic (252–66 MYA). Humphreys (2001a, 2008) further suggested that the marine transgression responsible for the Pilbara upland populations occurred in the Eocene (56–33.9 MYA), perhaps around 48 MYA (Sluijs et al. 2008).

Our results suggest that neither the Pilbara upland populations (*H.* sp. PH) nor the entire Australian *Halosbaena* lineage are likely old enough to have achieved their current distributions during the Eocene. If so, what could account for the presence of *Halosbaena* sp. PH at high elevation? The first and most obvious explanation is that the molecular dating analyses are inaccurate. This is certainly possible, although we were quite conservative in our assumptions, and our results generally make sense relative to other geological events. Further, the geological interpretations for the area, which sets the scene for our biological actors, have at least as many caveats, sources of error and unknown data.

One possibility is that the Pilbara uplands used to be lowlands, and geological uplifts of the whole landscape raised *Halosbaena* sp. PH to their current heights relatively recently. Uplifts through significant tectonic activity are known to have occurred in the area within the time frames suggested by our

data for the Pilbara High populations at both the Cape Range and Barrow Island, and especially in the basin around Exmouth Gulf (Kendrick et al. 1991). This very process may have raised the melitid amphipod populations found high up within the Cape Range itself, as this is a primarily marine family (Adams and Humphreys 1993). Haig and Mory (2003) suggest an uplift occurred at the Giralia Range (east of the Cape Range) of about 130–150 m after the Eocene, although this would not be quite sufficient to raise the *Halosbaena* sp. PH populations to their current elevation from the present-day coastline.

Another possibility is that *Halosbaena* migrated upstream from low to high elevation up alluvial river valleys by moving through the hyporheic zone beneath the river channel (Humphreys 2001a). *Halosbaena* occur throughout the length of the lower Robe River Quaternary alluvium aquifer (WFH data). There is no clear break that would preclude them colonising along the aquifer. However, other rivers draining the region flow through one or more water gaps due to incision of a superimposed drainage into the Precambrian base (Kriewaldt and Ryan 1967). The Proterozoic bedrock at the water gaps is effectively a hydrological boundary to the west (Barnett and Commander 1985). If this is the case on the Robe as it is on the Fortescue, direct colonisation from the coast by *Halosbaena* sp. PH to Pilbara uplands would be precluded. The ability of their migration along rivers is challenged by the situation in the Western Fortescue Plain aquifer. The Robe River now arises on the Western Fortescue Plain aquifer and is separated from the Fortescue River by a groundwater divide at 116.825°E (Barnett and Commander 1985), which should not, of itself, provide an impediment to the movement of stygofauna. Nonetheless, distinct faunal elements occur in the Robe and Fortescue sections of the aquifer, respectively, represented by Thermosbaenacea and Spelaeogriphacea. The present sample sites are all in Quaternary alluvia and so the ancestral populations must have migrated through different geologies over time, hampering our ability to interpret the actual landscape barriers and passages that would have confronted them.

A further possibility is that *Halosbaena* sp. PH were indeed facilitated to their current distribution in the Robe by a marine transgression; however, not one in the Eocene but later. A global sea-level high stand of about 150 m occurred between 7–4 MYA (derived from Miller et al. 2011, fig. 6). At first pass, this is not sufficient amplitude to account for the higher location of *Halosbaena* sp. PH in the Pilbara. However, consideration of the geology suggests otherwise. The Robe River follows the Robe Palaeovalley which is filled with goethite pisolites which were deposited 18–5 MYA (Heim et al. 2006). The palaeovalleys are incised deeply into the Proterozoic bedrock down to 140 m (to the top of the Banded Iron Formation) (Aquaterra 2005) and so there was groundwater and suitable habitat at an elevation of about 140 m around 5 MYA.

A simple, clean stranding of a coastal population by a single marine transgression is intellectually satisfying, but may not reflect a richer reality. Indeed, the “two step model”, by definition, requires two separate events. The geology of the Robe reveals that the habitable zone of *Halosbaena* sp. PH was as low as 140 m about 5 MYA, within the inferred age of the lineage (Fig. 4). This elevation could have been reached by its biological denizens by either the geological uplift suggested by Haig and Mory (2003) or the marine transgression inferred from Miller et al. (2011), with subsequent migration beneath the channel, or even a complex combination of all of these processes. Further longitudinal biological and geological sampling along the Robe River may resolve these competing interpretations.

Western Cape Range *Halosbaena* phylogeography

We have presented data to suggest species-level divergences within NWA *Halosbaena*, but large divergences presumably start as small ones. Smaller, geographically-based, within-species divergences are also evident within some of the Australian *Halosbaena* putative species identified here. There are genetic breaks of 1.3–3.6 % (COI) between some groups of sites on Barrow Island (Fig. S15), principally between eastern site GW05, northern site X62m, and a group of five central-eastern sites. There is geographic overlap between some of the groups, and the genetic landscape of Barrow Island is poorly known for other subterranean species, so this must remain just a potentially interesting phylogeographic pattern for now.

However, there are clearly defined breaks of 5.6–6.1 % between *H. tulki* populations within the continuous, coastal plain of the western Cape Range, which form northern, central and southern groups (Fig. 5). Given the marked divergences, both to the north and south from C-215 (central group), which is the type locality of *H. tulki*, they could well be separate species themselves. Here, we treat these divergences as deep phylogeographic breaks since our nuclear data are unclear and our sampling is inadequate to discriminate between clinal and vicariant structure. Our on-going complementary taxonomic treatment (King et al., unpublished data) of these populations may resolve this.

A similar pattern of distribution along this coastline is also found in other sympatric subterranean species, such as the atyid shrimp *Stygiocaris*, represented there by two species (Adams and Humphreys 1993; Page et al. 2008), and for the blind cave gudgeon, *Milyeringa*, with two distinct genetic populations (Adams and Humphreys 1993; Chakrabarty 2010; Larson et al. 2013). Interestingly, in both these two genera, the eastern population wraps around the northern point of the peninsula, and in both cases overlap at a northern site, occurring there in sympatry. *Stygiocaris* occurs as a separate species at the far southern site (Page et al. 2008). There are

even northern, central and southern genetic groupings within subterranean species higher up in the Cape Range itself, both for stygobites (melitid amphipod; Adams and Humphreys 1993) and troglobites (millipede; Humphreys and Adams 2001).

When geographic patterns of genetic structuring start to repeat themselves between unrelated, sympatric species, there is the hint of a large-scale geological process of some sort structuring the whole community (Page and Hughes 2014). Some are fairly obvious, such as the range separating the east and west coasts of the Cape Range peninsula mentioned above. However, the causes of the disjunctions in the apparently continuous habitat along the coastal plain is unknown; nonetheless, long-term gene flow amongst these populations of *Halosbaena* is severely restricted along the west coast (Fig. 5). The different responses and levels of divergence between taxa may be due to differences in the salinity tolerances of the various genera, for *Milyeringa* occurs in both freshwater and seawater (Humphreys 2001b), whereas *Halosbaena* is here largely confined to fresh or slightly brackish water. Numerous gorges in the range discharge across the coastal plain, where they form alluvial fans comprising coarse gravels/cobbles and conduits for freshwater storage and outflow. Humphreys and Adams (1991) postulated a salinity model for the coastal groundwater whereby salt water intruded inland between the alluvial fans and, while not restricting movement of the euryhaline *Milyeringa* along the coastal plain as much, may impede such movement by more freshwater-inclined fauna, such as *H. tulki*. Full multi-species analyses combined with further geological work should shed light on this “cryptic biogeography” (Page and Hughes 2014).

Conclusion

Our analyses have provided insights into evolutionary and biogeographic relationships at multiple scales, from across the globe down to very localised patterns between sites on a dusty, coastal plain in Western Australia. However, it is not clear how generalisable and “essential” (Winsor 2006) these patterns may be, since we have viewed them from only a single perspective, that of the Thermosbaenacea. The relationship between landscape and biota is not static, both dancing through time like a waltzing couple, sometimes splitting off to join another, and at other times sticking resolutely with their long-term partner. These moving targets are not easily interpreted in isolation, so to distinguish idiosyncratic, and yet still fascinating, stories from more general patterns, lots of dancers need to be watched carefully and simultaneously, and then compared (Page and Hughes 2014). In the current study, we have viewed multiple scales from one viewpoint,

but we also need the reverse, that is to view a single scale from multiple viewpoints.

There is an increasing build-up of single taxon subterranean studies (including this one), from all of the areas visited in our study. And yet, if we are truly serious about getting out of our “cave” and understanding the deeper processes responsible for these fascinating shadows of evolution worldwide, we need to carry out systematic, multi-species studies using modern genetic data and analyses at all levels in much the same way that Adams and Humphreys (1993) began more than 20 years ago using allozymes to understand numerous subterranean species of the Cape Range.

Acknowledgments We thank Garth Humphreys and Jason Alexander (Biota Environmental Sciences), Tom Iliffe (Texas A&M University), Alejandro Martínez García (University of Copenhagen) and Michitaka Shimomura (Kitakyushu Museum of Natural History) for access to specimens. Darren Brooks, Julianne Waldo and Rae Young provided support in the field, and Kylie Webster and Biota Environmental Sciences prepared our map. Cameron Schulz and Kate Hodges (Queensland Dept. of Science, Information Technology and Innovation) made useful comments on an earlier version of this manuscript. Matt Phillips (Queensland University of Technology) helped us to tame the BEAST, and attendees at the 2014 Broken Head Genetics Meeting on North Stradbroke Island provided advice on molecular dating. Two anonymous reviewers helped to strengthen this manuscript. All images and maps used with permission. Funding for this project came from the Western Australian Museum and Australian Rivers Institute, Griffith University. This work is dedicated to the memory of Rupert Page, who helped the first author to understand the underlying chemistry of a molecular laboratory, and, more importantly, that there is beauty in science.

Compliance with ethical standards

Ethics statement All methods comply with the current laws of Australia, where we did this research.

Conflict of interest There are no known conflicts of interest.

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