



Phylogeographic structure across one of the largest intact tropical savannahs: Molecular and morphological analysis of Australia's iconic frilled lizard *Chlamydosaurus kingii*



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ABSTRACT

The spectacular threat display of the savannah specialist Australo-Papuan frilled lizards has made them one of the world's most iconic reptiles. They are increasingly used as a model system for research in evolutionary biology and ecology but little is known of their population structure. Their distribution across northern Australia and southern New Guinea also provides an opportunity to examine biogeographic patterns as they relate to the large-scale movement of savannah habitat during the Plio/Pleistocene and the associated increase in aridity. We generated sequence data for one mitochondrial and four nuclear DNA loci (5052 base pairs) for 83 frilled lizards sampled throughout their range. We also quantified body proportion variation for 279 individuals. Phylogenetic analyses based on maximum likelihood and Bayesian species-tree methods revealed three shallow clades that replace each other across the monsoon tropics. We found the expected pattern of male biased sexual size dimorphism in both maximum body size and head size but there was no sexual dimorphism in overall body shape or in frill size, relative to head size, supporting the hypothesis that the frill is used primarily as a threat display rather than a sexual display. The genetic clades are broadly consistent with known clinal variation in frill color that gradually shifts from west to east (red, orange, yellow/white) but otherwise show little morphological differentiation in body proportion measures. The biogeographic breaks between clades occur at the Carpentaria Gap and the lowlands surrounding the Ord River, and our ecological niche modeling predicts lower habitat suitability for *C. kingii* in these regions. While this biogeographic pattern is consistent with numerous other taxonomic groups in northern Australia, the overall low genetic diversity in frilled lizards across the entire monsoon tropics and southern New Guinea contrasts starkly to patterns seen in other terrestrial vertebrates. Extremely low intra-clade genetic diversity over vast geographic areas is indicative of recent gene flow that would likely have been facilitated by widespread savannah during interglacials, or alternatively may reflect population bottlenecks induced by extreme aridity during Pleistocene glacials. The shallow divergence between Australian and New Guinean samples is consistent with recent connectivity between Australia and New Guinea that would have been possible via a savannah corridor across the Torres Strait. Based on our molecular and morphological data, we do not support taxonomic recognition of any of the frilled lizard clades and instead consider *C. kingii* a single species with shallow phylogeographic structure and clinal variation in frill color.

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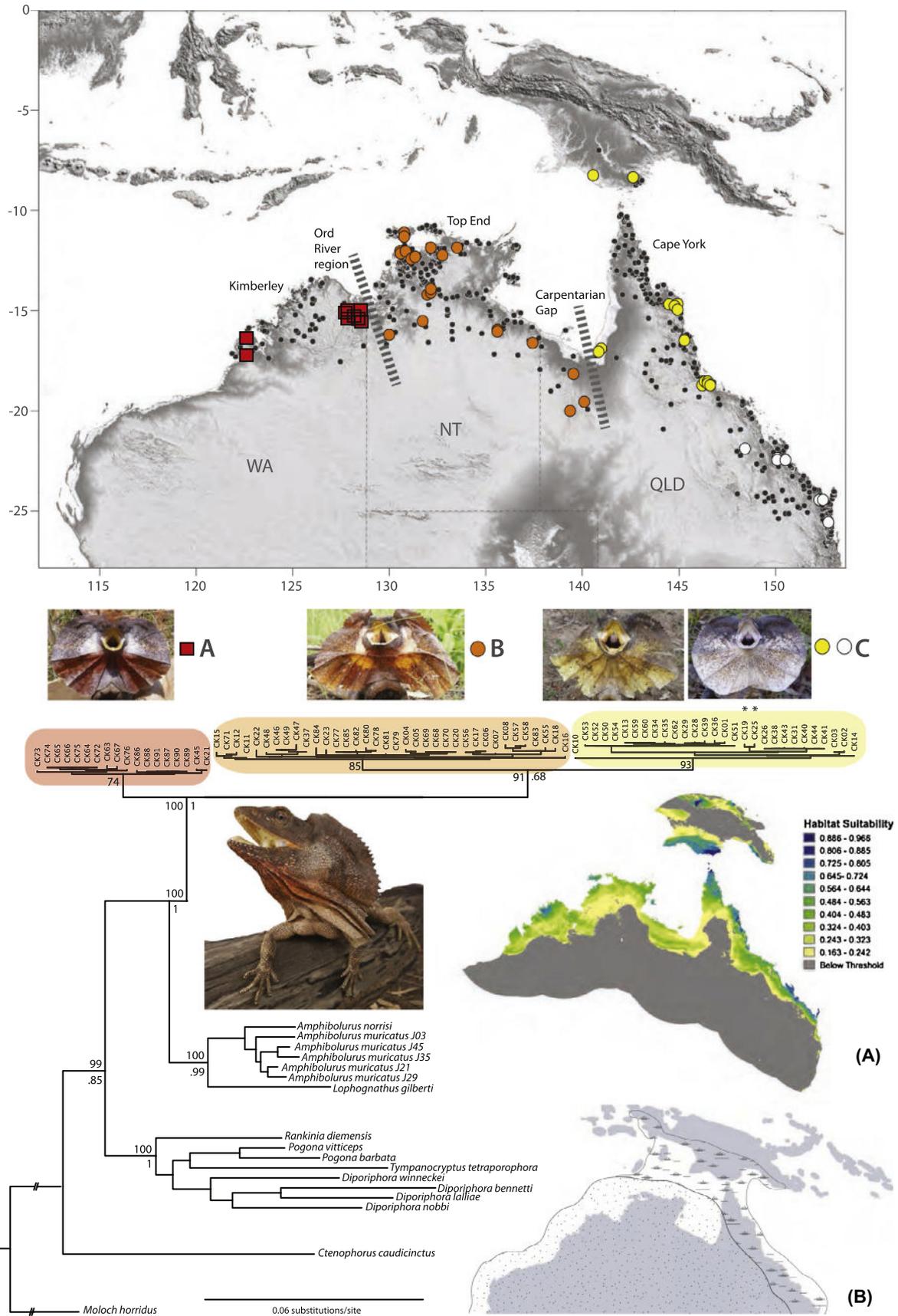
1. Introduction

In Australia the vast tropical savannah presently spans the full east-west extent of the northern third of the continent, and repre-

sents one of the largest relatively intact tropical savannahs on the planet (Bowman et al., 2010). This ecoregion extends across the Arafura Sea into the Trans-Fly savannah of southern New Guinea (Hill et al., 2010). Savannah ecosystems are thought to have originated in the Miocene (Pennington et al., 2006), concomitant with global cooling and a drying of climate (Flower and Kennett, 1994). Fossil evidence suggests grass-rich habitats were established in the mid-Miocene, with C4 grasses that characterize the

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savannah vegetation rapidly appearing ~8 Ma and becoming dominant by 4 Ma (Pennington et al., 2006; Beerling and Osborne, 2006). It has been suggested that during Pleistocene glacial cycles, the Australian savannah moved northward by up to 800 km in response to arid conditions (van der Kaars et al., 2000; Ford and Blair, 2005), with low sea levels during these times providing a corridor of savannah-type habitat periodically connecting north-eastern Australia with the island of New Guinea (Fig. 1).

There are many species adapted to the savannah ecosystem, including ecologically specialized endemics as well as geographically widespread taxa. The latter includes the iconic frilled agamid lizard, *Chlamydosaurus kingii*. The sole member of its genus, the frilled lizard was the first of the three most distinctive Australian agamids described by John Edward Grey in 1825 (followed by the thorny devil *Moloch horridus* and later the chameleon Dragon *Chelosania brunnea* [Witten, 1993]). Frilled lizards are among the largest Australian agamids reaching 90 cm in total length, and are characterized by an enormous fold of skin or 'frill' which is kept folded back against the body but can be erected to a diameter more than four times that of the lizard's body during threat displays and social interactions (Shine, 1990). Relative to body size, this is one of the largest and most spectacular display structures seen in the animal kingdom (Shine, 1990).

Frilled lizards are abundant throughout their distribution across the tropical savannahs of northern Australia and southern New Guinea, and as such have become a popular study system for exploring diverse evolutionary questions relating to complex visual signals and behavior (Shine, 1990; Christian et al., 1999; Hamilton et al., 2013; Merklung et al., 2015), reproductive ecology (Bedford et al., 1993; Harlow and Shine, 1999), physiology (Shine, 1990; Christian and Green, 1994; Christian and Bedford, 1995a; Frappell and Mortola, 1998; Christian et al., 1996, 1999), parasite ecology (Jones, 1994; Christian and Bedford, 1995b; Griffiths et al., 1998), and other aspects of ecology and fire management (Shine and Lambeck, 1989; Christian et al., 1995; Griffiths and Christian, 1996a, 1996b; Griffiths, 1999; Brook and Griffiths, 2004; Ujvari et al., 2011, 2015).

Recent work on color and signal function has revealed phenotypic variation in frilled lizard populations that may be indicative of underlying genetic differentiation. For example, frill color has been shown to vary geographically (Merklung et al., 2015); populations in Western Australia and the Northern Territory typically display a red or orange frill, while populations in eastern Queensland predominantly display a yellow or white frill. These different frill colors are produced by the interaction and variation of two discrete pigmentary mechanisms (carotenoids and pteridines) (Hamilton et al., 2013; Merklung et al., 2015), with carotenoid concentrations strongly decreasing from west to east, and with pteridines only present in the red and orange forms (Merklung et al., 2015). While molecular studies have assessed local gene flow at the population level (Ujvari et al., 2008), and three complete mitochondrial genomes have been sequenced (Ujvari and Madsen, 2008), no phylogenetic hypothesis of relationships among *C. kingii* populations from

throughout their broad range has been available for interpreting these data in an evolutionary context.

The distribution of frilled lizards across northern Australia and southern New Guinea make them an ideal system for further illuminating the biogeography the monsoon tropics – one of Australia's least explored biomes but now the center of intense research effort (Moritz et al., 2013). Northern Australia is broadly divided into three areas of endemism, each comprising major sandstone escarpments; the Kimberley Plateau, Arnhem Land, and the Cape York Peninsula (Cracraft, 1991). The ranges of Cape York in Queensland are separated from the Northern Territory by extensive low-lying clay plains of the Carpentaria Gap, while the sandstone plateaus of the Kimberley and the Top End are separated by low-lying landscapes associated with the floodplains of the Ord River basin, and to some extent the plains of the Daley and Victoria rivers (Fig. 1). Accordingly, biogeographic breaks in several taxa across the monsoon tropics (reviewed in Bowman et al., 2010; Eldridge et al., 2011; Catullo et al., 2014) coincide with these topographic discontinuities. Many recent studies on saxicoline taxa (Fujita et al., 2010; Oliver et al., 2010, 2013a, 2013b, 2014, P.M. Oliver et al., 2014; P. Oliver et al., 2014; Pepper et al., 2013; Doughty et al., 2012; Potter et al., 2012, 2016; Köhler and Burghardt, 2016; Köhler and Criscione, 2015; Moritz et al., 2016), repeatedly have revealed extraordinarily deep and localized diversity across the monsoon tropics. This biogeographic pattern is thought to have evolved through repeated isolation in mesic refugia provided by topographic uplands, that facilitated the persistence and diversification of lineages through periods of enhanced aridity. Conversely, the more extensive distribution of the savannah during glacial cycles should have promoted higher levels of connectivity and wider distributions for generalist savannah taxa (Bowman et al., 2010). This hypothesis is somewhat supported by recent studies of other monsoonal savannah taxa that typically display lower diversity and younger clade ages compared to rocky endemics, though substantial phylogeographic structure is evident nevertheless (Smith et al., 2011; Melville et al., 2011; Catullo and Keogh, 2014; Eldridge et al., 2014).

Here we present the first comprehensive phylogeny for the frilled lizard based on five loci, using samples from museums as well as our own recent field collections. In addition, we analyze morphological variation, and use environmental niche modeling to characterize suitable habitat across their current distribution. We compare our results to published studies of other taxa to assess the history of species diversification and historical biogeography across the savannahs of northern Australia.

2. Materials and methods

2.1. Genetic analyses

2.1.1. Taxonomic sampling

We sequenced data from 81 Australian *C. kingii*, and two samples from southern New Guinea (Table 1 and Fig. 1). Specimens

Fig. 1. Phylogeny of 83 *Chlamydosaurus kingii* samples and outgroups based on the combined *nd2*, *bach1*, *rag1*, *bdnf* and *prlr* data, and their distribution across Australia and New Guinea, overlain onto a digital elevation model image (Shuttle Radar Topography Mission) where light grey equates to areas of high elevation, and dark grey equates to areas of low elevation. Numbers above or on the left of nodes on the tree refer to ML bootstrap support from the RAxML analysis, while numbers below or on the right of nodes indicate posterior probabilities from the BEAST analysis. The samples from New Guinea are indicated in the phylogeny by an asterisk. Colored dots on the map refer to genotyped samples of the three *C. kingii* clades and their associated frill color, whereas small black dots refer to locality records of *C. kingii* downloaded from the Atlas of Living Australia. Thin dashed lines on the map represent State boundaries. WA = Western Australia, NT = Northern Territory, QLD = Queensland. Thick dashed lines refer to biogeographic barriers mentioned in the text (images of frill colors: D. Hamilton & B. Cser, central image of *C. kingii*: S. Macdonald). Inset A: Species distribution model of frilled lizards in Australia and New Guinea. Colors represent the predicted habitat suitability from high (blue) to low (yellow). Grey areas represent areas with suitability below the threshold value (see text for details). Inset B: Distribution of vegetation and exposed sea-floor during the last glacial (~18 ka) modified from Ford and Blair (2005) [based on van der Kaars et al., 2000]. Dots indicate where grassland, shrub and desert-type habitats were dominant, while the dashed pattern indicates where eucalyptus savannah-type habitat was dominant. Solid grey indicates the current coastline. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Museum accession numbers and collection locality information for all *C. kingii* sampled in this study. MV = Museum Victoria, WAM = Western Australian Museum, SAM = South Australian Museum, QM = Queensland Museum, Pryke = Personal collection of Sarah Pryke.

CLADE	LAB#	Tissue registration Number	Voucher registration number	Institution	State/Territory	Locality	Latitude	Longitude
A	CK21	14615	14615	MV	WA	Great Northern Highway, entrance to Kilo Station	-17.81583	122.67889
A	CK45	151776	151776	WAM	WA	Beagle Bay	-17.07660	122.70722
A	CK63	KF20 24/11	No voucher	Pryke	WA	Kununurra	-15.74522	128.64522
A	CK64	KM5 13/12PM	No voucher	Pryke	WA	Kununurra	-15.77006	128.73020
A	CK65	KM3 1/1AM	No voucher	Pryke	WA	Kununurra	-15.77026	128.73019
A	CK66	KM28	No voucher	Pryke	WA	Kununurra	-15.85066	128.77022
A	CK67	KM29 2/12	No voucher	Pryke	WA	Kununurra	-15.85320	128.75958
A	CK72	OM8 25/11	No voucher	Pryke	WA	Ord	-15.48109	128.86859
A	CK73	OM6 22/11	No voucher	Pryke	WA	Ord	-15.48145	128.86812
A	CK74	OM5 22/11	No voucher	Pryke	WA	Ord	-15.48168	128.86787
A	CK75	OM2 21/11	No voucher	Pryke	WA	Ord	-15.48341	128.86543
A	CK76	OF11 28/11	No voucher	Pryke	WA	Ord	-15.48442	128.86274
A	CK86	WM06 10/12 AM	No voucher	Pryke	WA	Wyndham	-15.45099	128.12005
A	CK87	WM5 11/12AM	No voucher	Pryke	WA	Wyndham	-15.45100	128.12007
A	CK88	WF51 12/12	No voucher	Pryke	WA	Wyndham	-15.47777	128.12554
A	CK89	WM60 13/12	No voucher	Pryke	WA	Wyndham	-15.48078	128.13341
A	CK90	WF42 9/12	No voucher	Pryke	WA	Wyndham	-15.86530	128.26448
A	CK91	WF47 10/12	No voucher	Pryke	WA	Wyndham	-15.86680	128.25612
B	CK04	11047	AMSR104187	SAM	NT	Kapalga	-12.67250	132.83234
B	CK05	106119	SAMAR55966	SAM	NT	Captive, parents ex Darwin area	-12.46242	130.84166
B	CK06	106120	SAMAR55967	SAM	NT	Captive, parents ex Darwin area	-12.46242	130.84166
B	CK07	13452	13452	SAM	NT	Gunn Point Road	-12.45165	131.09673
B	CK08	13453	13453	SAM	NT	Gunn Point Road	-12.45165	131.09673
B	CK11	102824	No voucher	SAM	QLD	Burke Developmental Road	-20.30782	140.24407
B	CK12	102862	No voucher	SAM	QLD	Wills Developmental Road	-18.82336	139.83384
B	CK15	30007	NTMR23039	SAM	NT	Goose Creek, Melville Island	-11.50889	130.90528
B	CK16	30050	NTMR22981	SAM	NT	20k SE Pickertaramoor, Melville Island	-11.90861	130.94861
B	CK17	30164	NTMR23109	SAM	NT	Darwin	-12.36667	130.87072
B	CK18	30592	NTMR23782	SAM	NT	Wickham River, Gregory NP	-16.81861	130.17417
B	CK20	53300	NTMR10933	SAM	NT	Near Alligator Head	-12.17500	132.24041
B	CK22	18827	18827	MV	NT	West Arnhem Land, Gubjekbinj	-12.25250	133.80194
B	CK23	18966	18966	MV	NT	100.7 km S of Willaroo turnoff, Buntine Highway	-16.05972	131.96278
B	CK37	A002980	J83903	QM	NT	Echo Point	-17.21000	137.78389
B	CK46	CF4 21/1	No voucher	Pryke	NT	Cape Crawford	-16.58248	135.92283
B	CK47	CF3 21/1	No voucher	Pryke	NT	Cape Crawford	-16.58944	135.90344
B	CK48	CM2	No voucher	Pryke	NT	Cape Crawford	-16.59766	135.88542
B	CK49	CF1	No voucher	Pryke	NT	Cape Crawford	-16.59810	135.88447
B	CK55	DM5 20/12	No voucher	Pryke	NT	Darwin	-12.33716	130.90181
B	CK56	DM6 20/12	No voucher	Pryke	NT	Darwin	-12.35741	130.87072
B	CK57	DF7 20/12	No voucher	Pryke	NT	Darwin	-12.35743	130.87052
B	CK58	DM2 19/12	No voucher	Pryke	NT	Darwin	-12.43578	131.13780
B	CK68	DM18 26/1	No voucher	Pryke	NT	Marakai Road, Darwin	-12.77206	131.45942
B	CK69	DM19 26/1	No voucher	Pryke	NT	Marakai Road, Darwin	-12.77675	131.45301
B	CK70	DM20 26/1	No voucher	Pryke	NT	Marakai Road, Darwin	-12.81204	131.42922
B	CK71	IM1	No voucher	Pryke	QLD	Mt Isa	-20.76296	139.48463
B	CK77	NM3 23/1	No voucher	Pryke	NT	Stuart Hwy; Katherine	-14.48577	132.40092
B	CK78	NF2 23/1	No voucher	Pryke	NT	Stuart Hwy; Katherine	-14.48789	132.40517
B	CK79	NM5 23/1	No voucher	Pryke	NT	Stuart Hwy; Katherine	-14.48823	132.40660
B	CK80	NM11	No voucher	Pryke	NT	Stuart Hwy; Katherine	-14.51365	132.42856
B	CK81	NM15	No voucher	Pryke	NT	Stuart Hwy; Katherine	-14.51683	132.42909
B	CK82	NM4 23/1	No voucher	Pryke	NT	Stuart Hwy; Katherine	-14.51749	132.42918
B	CK83	NM6 23/1	No voucher	Pryke	NT	Victoria Hwy, Katherine	-14.62010	132.13341
B	CK84	NM9 23/1	No voucher	Pryke	NT	Victoria Hwy, Katherine	-14.62848	132.12799
B	CK85	NM7 23/1	No voucher	Pryke	NT	Victoria Hwy, Katherine	-14.63239	132.12600
C	CK19	49510	AMSR121182	SAM	Papua New Guinea	Wipim	-8.78809	142.88238
C	CK25	A007736	No voucher	QM	West Papua	Wasur NP, 40 km E Merauke	-8.59952	140.83852
C	CK01	75998	75998	SAM	QLD	Davies Creek	-17.00704	145.57758
C	CK02	75999	75999	SAM	QLD	Davies Creek	-17.00704	145.57758
C	CK03	76000	76000	SAM	QLD	Davies Creek	-17.00704	145.57758
C	CK10	102776	102776	SAM	QLD	35k E Normanton	-17.67179	141.08973
C	CK13	16384	SAMAR34531	SAM	QLD	Townsville	-19.26667	146.81667
C	CK14	72765	SAMAR54347	SAM	QLD	12 Mile Creek Crossing on Burke Development Road	-17.51667	141.15944
C	CK26	A008059	J91148	QM	QLD	Barrett's Lagoon, Endeavour Valley Rd	-15.41667	145.15000
C	CK28	A008147	J91303	QM	QLD	Howard	-25.31667	152.55000
C	CK29	A008148	J91304	QM	QLD	Toogoom	-25.25000	152.71667
C	CK31	A009195	J91912	QM	QLD	Mary Vale, 18 km W Cooktown	-15.42972	145.11889
C	CK34	A001282	J82419	QM	QLD	Townsville area	-19.25000	146.81667

Table 1 (continued)

CLADE	LAB#	Tissue registration Number	Voucher registration number	Institution	State/Territory	Locality	Latitude	Longitude
C	CK35	A001850	J83132	QM	QLD	Gumlow Rd, Townsville area	−19.14889	146.67972
C	CK36	A003997	J83620	QM	QLD	Road to Nebo, 137 km N Dingo	−22.66889	148.67417
C	CK38	A003547	J84395	QM	QLD	Mary Vale, Jensen's Crossing, Endeavour Valley Rd	−15.42972	145.11889
C	CK39	A003728	J85279	QM	QLD	Lake Weyba, Noosa, western shore	−26.43333	153.06667
C	CK40	A005391	J87601	QM	QLD	Hope Vale, 1 km from, on Hope Vale Road	−15.28333	145.10000
C	CK41	A005544	J88033	QM	QLD	Little Annan Crossing, Lakeland-Cooktown Road	−15.11667	145.23333
C	CK43	A006869	J89060	QM	QLD	Oakey Creek turnoff, Endeavour Valley Rd	−15.48333	145.18333
C	CK44	A007428	J89695	QM	QLD	Battlecamp Rd, 40 km W Cooktown	−15.26667	144.81667
C	CK50	QF1	No voucher	Pryke	QLD	Capricorn Caves	−23.15198	150.71448
C	CK51	QM8	No voucher	Pryke	QLD	Capricorn Caves	−23.15974	150.43700
C	CK52	QF11	No voucher	Pryke	QLD	Capricorn Caves	−23.15977	150.43648
C	CK53	QF2	No voucher	Pryke	QLD	Capricorn Caves	−23.16624	150.48584
C	CK54	QM3 11/1	No voucher	Pryke	QLD	Capricorn Caves	−23.17367	150.47565
C	CK59	TF9 17/1	No voucher	Pryke	QLD	Hervey's Range	−19.31543	146.58893
C	CK60	TF10 16/1	No voucher	Pryke	QLD	Hervey's Range	−19.31615	146.58178
C	CK62	TM12 17/1	No voucher	Pryke	QLD	Hervey's Range	−19.32213	146.53168

were collected from throughout the Australian monsoon tropics between 2012 and 2013, and the remaining samples were obtained for all unique localities that were available from the Queensland Museum (QM), Museum Victoria (MV), the Western Australian Museum (WAM) and the South Australian Museum (SAM). We also included Genbank data for 17 additional agamid taxa (see Appendix Table 1 for all Genbank material used in this study). We included six samples spanning the genetic diversity within the closely related *Amphibolurus muricatus* (Pepper et al., 2014) for comparison of genetic divergences within *C. kingii*.

2.1.2. DNA amplification and sequencing

For all 83 individuals, we collected new sequence data for one mitochondrial locus (*nd2*). In addition, for a subset of 44 samples we also sequenced four protein-coding nuclear loci (*rag1*, *bach1*, *bdnf* and *prlr*). Individuals were selected to maximise representation of mitochondrial genetic diversity and geographic space. For the ingroup taxa, the data matrix was 100% complete for *bdnf*, 91% complete for *rag1*, 87% complete for *prlr*, and 80% complete for *bach1*. All new sequences from this study are deposited on Genbank (accession numbers KX786698–KX786937).

Genomic DNA was extracted from approximately 1 mm³ of tissue (either a tail tip or liver sample stored in 100% ethanol) using a QIAGEN DNeasy Blood & Tissue plate kit. For PCR and sequencing protocols for the same loci see the study of Pepper et al. (2014) on a closely related agamid lizard (*Amphibolurus muricatus*). The PCR/Sequencing primer combinations used in this study are listed in Appendix Table 2.

Sequences were edited and contigs were assembled in SEQUENCHER v. 4.10.1 (Gene Codes Corporation). Alignment of sequences was first performed automatically using the software MUSCLE (Edgar, 2004), then refined by eye in Se-AL (Rambaut, 1996). The few heterozygotes found in the nDNA genes were coded as such in the alignments prior to analyses. Protein-coding regions were translated into amino acid sequences and were checked for internal stop codons and frame-shift mutations.

2.1.3. Analysis of sequence data

Phylogenetic analyses were conducted using maximum likelihood (ML) in RAXML-VI-HPC v7.0.4 (Stamatakis, 2006). We concatenated the data and partitioned the combined dataset by codon position (excluding the tRNA which was treated as a single partition). We then used PartitionFinder (Lanfear et al., 2012) to establish the best partitioning strategy for the analysis, using

linked branch lengths, RaxML model of evolution and selected the best model based on the Bayesian Information criterion (3 subset partitions; [nd2_pos1, nd2_pos2, tRNA] [nd2_pos3] [bdnf_pos1, bdnf_pos2, bdnf_pos3, rag1_pos1, rag1_pos2, rag1_pos3, bach1_pos1, bach1_pos2, bach1_pos3, prlr_pos1, prlr_pos2, prlr_pos3]). Using RAXML we then implemented the general time-reversible substitution model with gamma-distributed rates among sites (GTR+G), with the best ML tree determined using 20 distinct randomized Maximum Parsimony (MP) starting trees. Bootstrap support was determined using 1000 replicates. We used *Moloch horridus*, one of the most basal lineages of the Australian agamids (Hugall et al., 2008) as the outgroup. A TCS haplotype network also was generated for each of the nDNA loci using the PopART program (<http://popart.otago.ac.nz>) (Supp. Fig. 1).

In addition to our concatenated ML approach, we used the hierarchical model implemented in *BEAST v. 2.0.2 (Bouckaert et al., 2013), that co-estimates the species tree and all gene trees in one Bayesian MCMC analysis, to estimate the species tree phylogeny. This method requires *a priori* designation of species or groups, so we divided our data based on the three genetic clades identified in our concatenated likelihood analysis, along with the outgroup taxa that were all coded as separate species (we were missing outgroup data for *bach1*). Models for each gene were inferred using PartitionFinder (Lanfear et al., 2012) using linked branch lengths, beast model of evolution, under the Bayesian Information Criterion. For *nd2*, the HKY+I+G substitution model was chosen, for *rag1* the model selected was HKY+G, while for *bach1*, *bdnf* and *prlr* the HKY substitution model was chosen. We unlinked loci and substitution models, employed a strict-clock model, and used a Yule tree prior. We conducted two separate runs, with samples drawn every 10000 steps over a total of 100000000 steps, with the first 10% discarded as burn-in. Acceptable convergence to the stationary distribution was checked by inspecting the posterior samples using the diagnostic software Tracer v1.5 (Rambaut and Drummond, 2007). Effective sample sizes were >200 for all parameters. All runs produced the same topology with very similar posterior probabilities, so we combined runs to generate a single consensus tree.

2.2. Morphological analyses

We evaluated body proportion data from 279 individuals captured from throughout their range in Australia. We excluded sexually immature individuals (less than 175 mm snout-vent length),

which left 231 individuals for analysis. The following body size measurements were taken with electronic calipers to the nearest 0.1 mm: snout-vent length (SVL), tail length (TL) from the cloaca to the tip of complete tails, head length (HL) from the back of the jaw to the tip of the snout, head width (HW) at the widest part of the head, head height (HH) at the highest part of the head, hind-limb length (HLL) from the groin to the tip of the longest toe, trunk length (TL) from the axilla to the groin, and maximum frill size (FS) from the bony ridges on the top of the head to the furthest extremity of the frill in a line parallel to the main axis of the body. Animals were weighed with an electronic scale to nearest 0.1 g and sex was determined by the presence or absence of hemipenes.

The vast majority of our genotyped specimens were also in our morphological data set. However, we had many additional specimens in the morphological data set, so we assigned them each to a genetic clade based on the putative distribution of each clade (the morphological samples were all from very similar locations to our genotyped specimens). Our primary interest was the evaluation of body shape differences, if any, between the three well-supported and geographically structured genetic clades (see results). We evaluated intra and inter-clade morphological variation, including sexual size dimorphism on natural log transformed data. We tested for sexual size dimorphism (SSD) in individual morphological traits within and between clades. We used Principal Components Analysis (PCA) with variance-covariance, which does not identify groups *a priori*, and Discriminant Function Analysis (DFA), where the groups were specified *a priori*, to examine the patterns of relationship and discriminating power of the body proportion characters (natural log transformed) with the statistics software JMP v. 8.0. The first PC represented variation in body size and the second PC summarized shape differences. We performed DFA on the ln-transformed data to examine if body shape differences would be sufficient to distinguish genetic clades when they were specified *a priori*.

2.3. Environmental niche modeling

We used MaxEnt v. 3.3.3k (Phillips et al., 2006; Elith et al., 2011) to generate a species distribution model (SDM) for the frilled lizard. All distribution records for *C. kingii* were downloaded from the Atlas of Living Australia (ALA; www.ala.gov.au; 31 March 2015), combined with genetic localities, and duplicates removed from within each grid cell using R (v. 3.1.3; R Core Team, 2015). The species was modeled as a whole, versus individual clades, as it is more likely to represent the fundamental ecological limits of the species (see Warren et al., 2014). Out of 948 unique presence locations, we randomly subsampled 500 unique locations as our presence data set using the sample function in R. We included 5 environmental variables at a 1 km² grid resolution – minimum monthly temperature, maximum monthly temperature range, topographic wetness index, and minimum and maximum monthly atmospheric water deficit (T. Harwood, in prep). These layers were buffered to within 600 km of the full presence dataset and used for both model training and prediction. In order to minimize model overfitting, we used the multivariate analysis implemented in ArcMap (v. 10.2; ESRI, Redlands, CA, USA) to calculate Pearson's correlation coefficient (*r*) between each pair of variables, and ensured our variables were not highly correlated (*r* < 0.80). To calculate sampling bias, we downloaded all Australo-Papuan records for the Family agamidae from the ALA (31 March 2015), without *C. kingii*. We then combined all agamidae and *C. kingii* records, and removed any duplicates within a grid cell using R. Sampling bias was then calculated across the target region using the point density function in ArcMap. Our model settings were 5000 maximum iterations, convergence threshold = 0.00001,

prevalence = 0.5, 10,000 pseudoabsences, and subsampled using a 25% random test percent. One hundred replicates were generated, and the average result is presented.

3. Results

3.1. Phylogenetic analyses

Following the removal of ambiguously aligned nucleotide sites, the final *nd2* dataset consisted of 1363 base-pairs (bp), *bach1* consisted of 1208 bp, *rag1* consisted of 1192 bp, *bdnf* consisted of 714 bp, and *prlr* consisted of 575 bp, totaling 5052 bps for the concatenated dataset. The gene tree for *nd2* exhibited phylogeographic structure with well supported clades but with lower support for the deeper relationships among them, while the individual gene trees for the nuclear loci showed low variability, generally appearing as a single or a series of shallow combs with few variable sites.

Our results from the partitioned ML analyses support three shallow clades within *C. kingii*; one comprising samples from the Kimberley (Clade A), another comprising individuals from the Top End to the Carpentarian Gap (Clade B), and a third clade comprising individuals from Queensland east of the Carpentarian Gap, Cape York and New Guinea (Clade C) (Fig. 1). We recovered strong bootstrap support (100) for the Kimberley clade (West) being sister (average 5.9% uncorrected p distance in the mtDNA data) to all other samples, which are structured into two weakly diverged (average 2.8% uncorrected p distance in the mtDNA data) lineages (Central [Top End and western Queensland] and East [eastern Queensland and New Guinea]) (Fig. 1). Our *BEAST species tree analysis agreed with this topology, but with lower support for the grouping of the Central clade with the East clade (Posterior Probability (PP) = 0.68). Both our ML and Bayesian analyses strongly support *C. kingii* to be sister to a clade comprising *Amphibolurus muricatus* and *Lophognathus gilberti* (PP = 0.99, Bootstrap Support (BS) = 100).

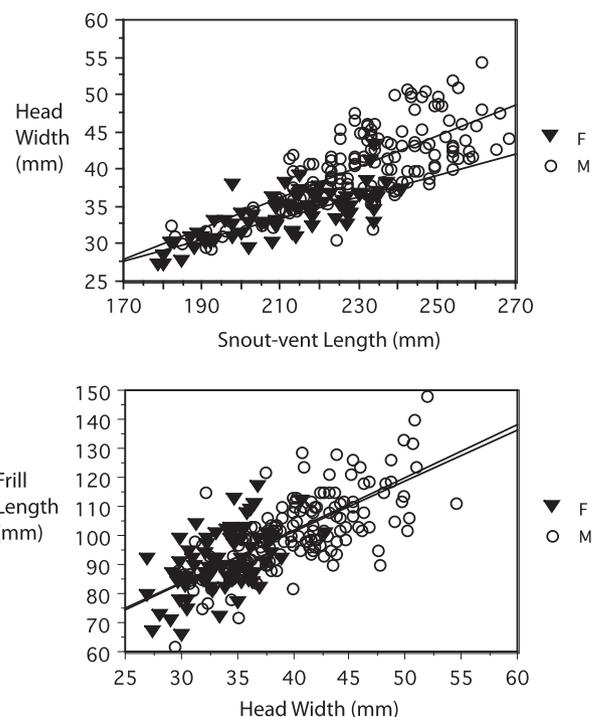


Fig. 2. Plots of key morphological traits. The top figure shows the difference in adult body size and head size dimorphism between male and female *Chlamydosaurus kingii*. The bottom figure shows that males and females have the same frill size, relative to head size.

3.2. Morphological analyses

The variation in body proportion data for adult males and females for each of the three genetic clades is summarized in Appendix 4. While Clade C had the largest individuals, all three clades displayed similar maximum SVLs for males and females, respectively, and the mean values and ranges for all the morphological traits measured were similar. Given this result, we evaluated sexual size dimorphism across the three genetic clades together. Males grow to larger maximum SVLs than do females and there also is sexual size dimorphism in head size measurements (Fig. 2). At the same adult SVL, males have longer heads than females (ANCOVA, slopes homogeneous $F_{1,226} = 2.66$, $P = 0.1042$; intercepts $F_{1,227} = 57.89$, $P < 0.0001$), but similar trunk lengths (ANCOVA, slopes homogeneous $F_{1,225} = 3.24$, $P = 0.0734$; intercepts $F_{1,226} = 0.21$, $P = 0.6486$). At the same head length, males have wider heads than females (ANCOVA, slopes homogeneous $F_{1,226} = 3.59$, $P = 0.0594$; intercepts $F_{1,227} = 2.49$, $P = 0.1161$). Frill size is more complex. At the same SVL, males have larger frills than females (ANCOVA, slopes homogeneous $F_{1,226} = 0.37$, $P = 0.5436$; intercepts $F_{1,227} = 7.34$, $P = 0.0072$), but at the same head length or width, there is no sexual size dimorphism in frill size (ANCOVA Head length, slopes homogeneous $F_{1,226} = 0.07$, $P = 0.7984$; intercepts $F_{1,227} = 0.56$, $P = 0.8123$; ANCOVA Head width, slopes homogeneous $F_{1,226} = 0.002$, $P = 0.9655$; intercepts $F_{1,227} = 0.08$, $P = 0.7768$). Thus, frill size is more strongly correlated with head size, rather than SVL, in both males ($R^2 = 0.49$) and females ($R^2 = 0.29$) yet is still highly variable.

We used all of the morphological data in PCA and DFA to evaluate differences, if any, between the genetic clades. PCA analyses demonstrate that overall body size (PC1, 76% of the variation) and shape (PC2, 9.7% of the variation) show both a great deal of variation and overlap across the three genetic clades (Fig. 3). PC2 loaded most strongly on tail and frill length. However, there is

evidence for subtle clinal variation in some traits. Clade C tended to have slightly smaller relative head and frill sizes than the other two clades, while Clade B tended to have slightly shorter relative tail and trunk lengths and slight longer relative hind-limb lengths. Relative head and frill sizes were among the most variable of the characters we measured. Despite this subtle variation, the amount of overlap between clades meant that DFA incorrectly allocated 23% of individuals to the wrong clade, which emphasizes the morphological homogeneity across the range of the species.

3.3. Environmental niche modeling

MaxEnt model predictions (Fig. 1, inset A) appeared to be accurate, with mean test data AUC of 0.8595 ± 0.0118 . The threshold value for our dataset was 0.1621 (maximum test sensitivity plus specificity logistic threshold, following Liu et al., 2013). Jackknife tests of variable importance indicate minimum monthly temperature and minimum monthly atmospheric water deficit are most important to model performance, suggesting that range edges are linked to the Australian arid regions and New Guinea highlands being too dry and/or cold for *C. kingii* (Appendix Table 3).

4. Discussion

We have produced the first phylogeny for populations of *Chlamydosaurus kingii* across its range, and present this genetic information alongside analyses of morphological variation and ecological niche modeling. Our data show strong support for three shallow genetic groups with allopatric distributions across northern Australia (Fig. 1). This genetic structure is supported by our environmental niche model that identifies lower habitat suitability in the geographic regions separating these clades. In addition our morphological data shows clinal variation in frill color, but reveals the frilled lizard is otherwise morphologically conservative across its range with only subtle variation in some body proportion measures. Below we discuss the taxonomic implications of our study, as well as the historical processes and biogeographic barriers that may have influenced the evolutionary history of this iconic savannah taxon.

4.1. Phylogeny and biogeography of *C. kingii* populations

Existing phylogenetic studies based on one or two genes have placed *C. kingii* within the *Amphibolurus* group of the Australian agamid radiation Amphibolurinae (Schulte et al., 2003; Hugall et al., 2008; Melville et al., 2011; Pyron et al., 2013), a clade also comprising the Jacky Dragon *Amphibolurus muricatus*, *A. norrisi* and *A. gilberti*. Our phylogeny, based on data from one mtDNA locus and four nuclear loci, also recovered this topology but the relationships within *C. kingii* were largely driven by the mtDNA data given the shallow divergence between the clades. For comparison, divergences between lineages of the closely related Jacky Dragon, *Amphibolurus muricatus*, using the same loci (Pepper et al., 2014) are very similar to those between *C. kingii* clades.

The phylogeographic patterns seen in *C. kingii* are largely concordant with numerous other monsoonal tropics taxa (e.g. Jennings and Edwards, 2005; Braby, 2008; Toon et al., 2010; Smith et al., 2011; Melville et al., 2011; Eldridge et al., 2014; Kearns et al., 2014; Potter et al., 2016), with genetic differentiation across the well-known biogeographic barriers of the Carpentaria Gap and the Ord River region (Fig. 1). This diversification pattern is likely associated with increased aridity in these regions compared to adjacent topographic uplands. Aridity in northern Australia is broadly linked to a weakening of the monsoon, and palaeoenvironmental evidence suggests that the effectiveness of

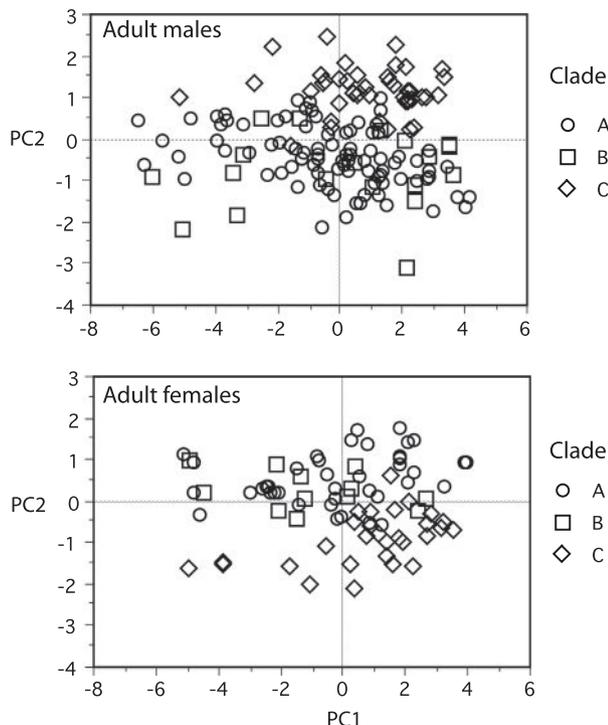


Fig. 3. PCA plots of adult male and female *Chlamydosaurus kingii* from across their range, with genetic clades shown. PCA based on analyses of ln transformed morphological data presented in Appendix 4 and demonstrate the large amount of overlap between genetic clades in body shape.

the monsoon was greatly reduced during the Last Glacial Maximum (LGM) (Fitzsimmons et al., 2013), creating significant aridity, causing lakes and rivers to dry, vegetation to become increasingly sparse, and sand dunes to become active (Hesse et al., 2004). In particular, drier conditions and cooler temperatures are thought to have been particularly pronounced in the lowlands (Reeves et al., 2013). In addition, the exposed continental shelf during glacial cycles greatly increased the distance to the coastline, that would further exacerbate the extreme climatic conditions in these regions.

Environmental niche modeling of *Uperoleia* frogs across the monsoon tropics Catullo et al. (2014) identified mean diurnal range (i.e. the difference between maximum daytime and minimum nighttime temperatures) as a major variable driving lineage divergence between areas of endemism, with the authors suggesting the cooler temperatures associated with increased aridity during glacial cycles of the Pleistocene provided barriers to dispersal for these savannah taxa. Our environmental niche model predicts habitat of lower suitability for *C. kingii* in the Gulf of Carpentaria as well as inland in the Top End, with this result being driven primarily by the ‘monthly minimum atmospheric water deficit’ (precipitation minus potential evaporation), followed by ‘minimum monthly temperature’ (Supplementary Table 3), highlighting the climatic limits of *C. kingii* also are defined by dry and cold conditions. These data support a hypothesis of Catullo et al. (2014) that minimum temperature, not just precipitation, is a major limiting factor for monsoonal taxa.

The shallow genetic divergence observed between *C. kingii* lineages, and furthermore the limited structure within clades over vast geographic distances, contrasts strongly to patterns seen in other lizards, that instead show deep divergences between these areas of endemism, as well as high levels of population divergence within them (Fujita et al., 2010; Pepper et al., 2013; Oliver et al., 2010, 2013a, 2013b, 2014, P.M. Oliver et al., 2014; P. Oliver et al., 2014; Moritz et al., 2013, in press). Recent studies in the Kimberley in particular have revealed cryptic diversity in the herpetofauna at a scale not seen anywhere else on the continent (e.g. Oliver et al., 2010, 2013a, 2013b; Pepper et al., 2011; Moritz et al., in press), while there appears to be little variation in *C. kingii* populations across this region (Fig. 1). Even taxa more broadly considered savannah generalists show greater lineage diversification and phylogeographic structure (Smith et al., 2011; Melville et al., 2011; Catullo and Keogh, 2014). This low level of genetic diversity within frilled lizards across the entire monsoon tropics region in northern Australian and southern New Guinea is indicative of recent population connectivity and gene flow that could have been facilitated by widespread and continuous savannah. Indeed, open savannah vegetation is thought to have connected the Malaysian Peninsula with the islands of Sumatra, Borneo and Java during the last glacial period (Bird et al., 2005), as well as New Guinea and Australia (van der Kaars et al., 2000). The basin of the Gulf of Carpentaria also was exposed for long periods during the Pleistocene, providing opportunities for dispersal between Cape York and the Top End (Ford and Blair, 2005). Ford and Blair (2005, Fig. 2, and see our Fig. 1) present a figure illustrating the distribution of savannah-type habitat in northern Australia and New Guinea during the last glacial, and suggest that while desert-type habitats dominated the monsoon tropics region at that time, as well as extending into the exposed dry land of the continental shelf, smaller areas of savannah habitat would also have been present. Given the ancestors of *C. kingii* were woodland-adapted organisms (Hugall et al., 2008), it is possible that increasing aridity and the expansion of semi-desert grasslands during periods of the Pliocene could have isolated *C. kingii* populations in small pockets of savannah-type habitat separate to the more continuous belt of savannah to the north (Ford and Blair, 2005), and driven population diversification into the three lineages

seen in *C. kingii*. Divergence could have been maintained during Pleistocene glacial cycles despite lowered sea-levels by cold and dry extremes in the lowlands of the Ord basin and the Gulf of Carpentaria, exacerbated by the increased distance to the climate-ameliorating coast.

The strong affinity between the fauna of Australia and New Guinea has long been recognised (Cogger and Heatwole, 1981), with these regions sharing a diverse array of taxa, especially at the generic level (Allison, 2006; Wüster et al., 2005; Menzies, 2006; Todd et al., 2014). Major sea-level fluctuations during glacial cycles of the Pleistocene periodically exposed the land-bridge between Australia and New Guinea, facilitating dispersal and connectivity between the landmasses, most recently at the LGM ~ 17 Ka ago. Even with sea levels as little as 10 m below present levels, New Guinea remained largely connected to Cape York across the Torres Strait (Voris, 2000). The two samples included in our study from southern New Guinea are nested well within the Queensland *C. kingii* clade (Fig. 1), suggesting populations were continuous until sea-levels rose following the LGM. This recent connectivity would have been facilitated by a narrow band of savannah-type habitat that is thought to have extended up the coast of eastern Australia to the Trans-Fly lowlands of New Guinea (van der Kaars et al., 2000; Ford and Blair, 2005). Our niche model also predicts environmental suitability for *C. kingii* in far eastern, as well as northern New Guinea, where there are no distribution records in the ALA database. While the savannah-woodland habitat in the eastern area appears suitable for *C. kingii* (E. Rittmeyer pers com), it seems unlikely this is a false absence due to lack of sampling, given this region includes Port Moresby, which is potentially the most well sampled area of New Guinea. The absence of *C. kingii* in this region is perhaps better explained by the current isolation of this suitable savannah-woodland from the more extensive Trans-Fly savannah by rainforest and swamp habitats. If populations of *C. kingii* colonized New Guinea very recently during the LGM, as our phylogeny appears to support, they may simply have not had time to disperse to eastern New Guinea before the savannah-woodland was fragmented by closed forest. Indeed, a rapid rise in sea level and temperature following the LGM lead to fluctuations in rainforests across New Guinea (Hope and Aplin, 2006), and archeological evidence from the nearby Aru islands in eastern Indonesia indicates a loss of savanna and grass-dwelling mammals around 7000 years ago (O’Connor et al., 2006). While environmental suitability also was predicted by our model in northern New Guinea, the rugged and topographically extreme central highlands likely prevented the northward dispersal of frilled lizards. Perhaps more importantly, there is an absence of suitable savannah-woodland in this region, which instead is dominated by lowland rainforest and swamp forest.

4.2. Morphological variation

Our morphological data demonstrate that *C. kingii* shows strong male-biased sexual size dimorphism in head size and maximum body size in all three clades, confirming results from a similar study based on smaller sample sizes (Shine, 1990), but body proportions are otherwise relatively conservative across the clades. The strength and direction of sexual size dimorphism, but the otherwise conservative morphology across clades, is similar to that found in the closely related Jacky Dragon, *Amphibolurus muricatus*, (Pepper et al., 2014). There is no sexual size dimorphism in frill size relative to head size. Instead frill size is tightly correlated with head size, and both sexes display a virtually identical allometric relationship between head and frill size (Shine, 1990). Shine (1990) evaluated a number of alternative hypotheses to explain the extreme size of the frill relative to other related dragon species and concluded, after extensive observation of wild animals, that

the frill is used primarily as a threat display rather than a sexual display.

While frill size is not sexually dimorphic, it is nonetheless quite variable between individuals and there is a high degree of overlap both within and between clades. Previous studies on frill color also have identified clinal variation across the range of the frilled lizard. Frills are redder in the west, grade to a more orange color in the central part of their distribution, become yellow in the east and then more white in southern part of their distribution in Queensland. Our genetic groups largely conform to this geographic variation in frill color, with the Kimberley “West” clade comprising individuals with a red frill, the Top End/western Qld “Central” clade comprising individuals with an orange frill, and the “East” clade comprising individuals from the remainder of the range in Queensland and southern New Guinea possessing a yellow or white frill. However, the relationship we recovered between clades did not match well with the differing pigmentary mechanisms for producing frill color. The red and orange hues are produced by high concentrations of carotenoids and pteridines (Merkling et al., 2015), but these forms are not sister taxa in our phylogeny. Instead the orange form is most closely related to the yellow/white forms, which have low carotenoid concentration and lack pteridines altogether (Merkling et al., 2015), suggesting frill color variation is not only due to genetic differences.

Carotenoids cannot be synthesised *de novo* but need to be acquired through diet (Olson and Owens, 1998). Hence it is possible that color differences have an environmental basis, and regional dietary differences have resulted in color differentiation, although further testing of this hypothesis is necessary. Alternatively, color variation may have evolved under selection. Two main hypotheses have been suggested to explain the evolution of the frilled lizard's frill and its color: sexual and predator threat display (Shine, 1990). Our morphological analyses found that frill size, relative to body size, did not differ between sexes, which is more consistent with the frill functioning as a predator threat display rather than a sexual display. Accordingly, no obvious sex differences were previously found in frill coloration (Merkling et al., 2015). If the frill predominantly functions as a predator deterrent, color variation may provide a selective advantage in different habitats. Despite being broadly classified as savannah, the habitat varies considerably across the geographic range of the frilled lizard, with the more open woodlands of northern Australia developed on the red earths contrasting to the wetter and comparatively more dense forests of the Queensland coast. Prey detection is likely to be easier in more open habitat, hence predation pressure may be greater in the red and orange forms and redder hues, often utilized as an aggressive signal (e.g. Healey et al., 2007; Little and Hill, 2007), may be beneficial.

In summary, our data demonstrate that the frilled lizard comprises three shallow allopatric clades that are only recently diverged. While the clades show clinal variation in frill color and subtle variation in frill size, there is considerable overlap in these characters and the clades are otherwise morphologically conservative. We do not support taxonomic recognition of any of the clades and instead consider *C. kingii* a single species with shallow phylogeographic structure.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymp.2016.09.002>.

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