

Supplementary material

Evidence for a single panmictic and genetically diverse population of the coconut crab *Birgus latro* (Decapoda: Anomura: Coenobitidae) on Christmas Island in the Indian Ocean

C. Anagnostou^{A,C} and *C. D. Schubart*^B

^AZoological Institute, Department of Evolutionary Ecology and Genetics,
Christian-Albrechts-University of Kiel, D-24118 Kiel, Germany.

^BZoologie und Evolutionsbiologie, Universität Regensburg,
D-93040 Regensburg, Germany.

^CCorresponding author. Email: canagnostou@zoologie.uni-kiel.de

DNA extraction

DNA extraction from haemolymph: after thawing, each 2-mL microtube containing 0.5 mL of haemolymph and 1.5 mL of 96% v/v extra pure ethanol was thoroughly vortexed to homogenise the denatured haemolymph and ethanol. A total of 1 mL of the haemolymph-ethanol mixture was distributed to two sterile 1.5-mL microtubes (500 μ L per microtube). The mixture was centrifuged at 10 000g for 30 s and the ethanol decanted. The microtubes containing the haemolymph pellet were placed under a fume hood for 10 min with open lids for excess ethanol evaporation. Subsequently, the protocol for DNA extraction given by Omega Bio-Tek was followed. To achieve a high yield, DNA was eluted twice with 50 μ L of the provided pre-warmed (70°C) elution buffer. The two eluates stemming from one haemolymph sample were transferred to a single sterile microtube before storing in the freezer at -20°C .

DNA extraction from muscle tissue: each dactylus was retrieved from the 15-mL falcon tube containing 10 mL of 96% v/v extra pure ethanol with heat-sterilised forceps. A small amount of tissue (~20–28 mg) was taken from within the dactylus, accessed by the cutting edge using heat-sterilised Dumont-forceps, and placed on the protected side of a piece of Parafilm M (Pechiney Plastic Packaging). The piece of tissue was teased using two heat-sterilised Dumont-forceps, and placed into a sterile 1.5-mL microtube (Eppendorf). Subsequently, the protocol for DNA extraction given by Macherey-Nagel was followed. To achieve a high yield, DNA was eluted twice with 50 μ L of the provided pre-warmed (70°C) elution buffer and stored in the freezer at -20°C .

Fragment analysis

In each well of a MicroAmp Optical 96-Well Reaction Plate, 11 μ L of Hi-Di Formamide, 0.15 μ L of GeneScan 400HD ROX Size Standard (Life Technologies), and 1.4 μ L (1.3 μ L for GBL08 & GBL09) of sterile and extra pure water were mixed. A total of 0.1 μ L of PCR product (0.2 μ L for GBL08 & GBL09) were added to each well, and the plate was covered with sealing tape (Sarstedt). After a brief centrifugation at 3500 rpm (equivalent to 2109g) at room temperature (23°C) for 30 s, the plate was transferred to the Institute for Clinical Molecular Biology (ICMB) at the Christian-Albrechts-University in Kiel, Germany for subsequent length-fragment analysis.

Table S1. Characteristics of the seven utilised microsatellite markers

T_a , annealing temperature; F, forward; R, reverse

GenBank Accession number	Locus	Repeat motif	Primer sequence (5'–3') and labelling	T_a (°C)
EU789581	GBL01	(GA) ₂₇	F: TTGAGACAAATAGTGTGTGCATTG- HEX R: AGCCACAATATCAGGGCACAAG	60
EU789582	GBL02	(GA) ₂₈	F: GGGTGAGGTAAAGGCTGCTGTG- FAM R: ACACTTAAAATGTTTGGCAGG	58
EU789583	GBL03	(GA) ₃₁	F: TGGTGTTTGAATTTGCATAACG- HEX R: GATGATGGGAAGCGACGAGG	57.6
EU789587	GBL07	(GT) ₃₈	F: TTGCATGTCTTGTGCCCTG- FAM R: TTTAATTTCGTTCCGGTCAGG	57.6
EU789590	GBL08	(GA) ₁₆ G(GA) ₁	F: GTGGATGCAGAGCCGTAGTCC- HEX R: TGTGGAAGACTCGTTTCCTCG	62
EU789589	GBL09	(TG) ₂₉ CG(TG) ₂₈	F: GATCCTGACCGACCCGG- FAM R: TCTTCCTCATTGCCAAGGTCG	60
– ^A	(TGGTG) ₄	(TGGTG) ₄	F: GCGCAAATTACGCTTCACC- HEX R: CACCTCTTCTACACCCGCGTC	60

^ANot yet listed in GenBank. Information about the marker kindly provided by Chai-Hsia Gan.

Table S2. Reagents and their respective volumes for a PCR Master Mix of a final volume of 10 µL per reaction

Reagent	Volume
Sterile, extra pure water	6.55 µL
5× MyTaq Reaction Buffer Colorless	2.00 µL
HEX- or FAM-labelled forward primer (concentration 20 µM)	0.20 µL
unlabelled reverse primer (concentration 20 µM)	0.20 µL
MyTaq HS polymerase (Bioline)	0.05 µL
template DNA (either non-diluted or 1 : 50 diluted eluate)	1 µL

Table S3. PCR profiles for the utilised microsatellite loci GBL01, GBL02, GBL03, GBL07, GBL08, GBL09 and (TGGTG)₄

PCR step	Temperature	Duration	Cycles
Denaturation	95°C	3 min	
Denaturation	95°C	30 s; (TGGTG) ₄ , 20 s	
Annealing	Locus-specific annealing temperature	30 s; (TGGTG) ₄ , 20 s	35; GBL07, 38
Extension	72°C	25 s; (TGGTG) ₄ , 15 s	
Final extension	72°C	1 min; GBL03, 25 s	

Table S4. Characteristics of the seven utilised microsatellite markers for individuals of *Birgus latro* from five sample sites on Christmas Island and 27 individuals of *B. latro* from Lanyu (Taiwan) investigated by Gan *et al.* (2008)

N_g , number of genotypes; N_a , number of alleles; H_o , observed heterozygosity; H_e , expected heterozygosity; bp, base pairs. Significant deviation from Hardy–Weinberg Equilibrium according to the procedure of Guo and Thompson (1992) with $\alpha = 0.05$ is indicated by asterisks. H_o and H_e were calculated using Arlequin, ver. 3.5.1.3 (Excoffier *et al.* 2005). Values of H_o , H_e and the P -value in parentheses were calculated with the dataset corrected for null alleles

Sample site	Locus	N_g	N_a	Allele size range (bp)	H_o	H_e	P -value
ECT	GBL01	20	13	151–187	0.90	0.927	0.41759
	GBL02	20	14	149–181	0.85	0.931	0.37378
	GBL03	20	16	75–135	0.70 (0.75)	0.880 (0.895)	0.00981* (0.04453*)
	GBL07	20	16	187–239	0.60	0.929	0.00520*
	GBL08	20	20	133–191	0.75	0.959	0.00027*
	GBL09	20	19	129–181	0.90	0.955	0.49797
	(TGGTG) ₄	20	12	445–620	0.75	0.912	0.02876*
DB	GBL01	20	16	151–191	0.75	0.922	0.00702*
	GBL02	20	15	149–181	0.70	0.919	0.00124*
	GBL03	20	17	75–137	0.85	0.882	0.59815
	GBL07	20	13	177–223	0.55 (0.65)	0.906 (0.921)	0.00000* (0.00120*)
	GBL08	20	17	133–181	0.90	0.942	0.15553
	GBL09	20	17	137–183	0.80	0.936	0.00103*
	(TGGTG) ₄	20	9	445–620	0.50 (0.75)	0.821 (0.864)	0.00044* (0.02910*)
HS	GBL01	20	13	151–187	0.75	0.903	0.14295
	GBL02	20	16	141–181	0.85	0.929	0.35896
	GBL03	20	13	75–139	0.50 (0.70)	0.851 (0.895)	0.00000* (0.00053*)
	GBL07	20	13	187–223	0.75	0.927	0.08441
	GBL08	20	17	133–197	0.60	0.953	0.00000*
	GBL09	20	18	133–171	0.95	0.951	0.59628
	(TGGTG) ₄	20	9	445–620	0.65 (0.70)	0.842 (0.855)	0.06208 (0.12229)
PH	GBL01	20	15	151–191	0.85	0.903	0.39601
	GBL02	20	17	147–185	0.70	0.935	0.00280*
	GBL03	20	13	75–139	0.55 (0.70)	0.820 (0.860)	0.00089* (0.04522*)
	GBL07	20	12	187–223	0.55 (0.70)	0.863 (0.895)	0.00000* (0.00782*)
	GBL08	20	21	133–189	0.95	0.963	0.32112
	GBL09	20	20	129–181	0.95	0.958	0.79086
	(TGGTG) ₄	20	9	445–620	0.80	0.845	0.38502
TD	GBL01	20	14	151–205	0.85	0.917	0.56188
	GBL02	20	18	149–189	0.80	0.940	0.06474
	GBL03	20	16	75–137	0.85	0.862	0.70483
	GBL07	20	15	187–241	0.85	0.938	0.25618
	GBL08	20	18	133–179	0.70	0.951	0.00013*
	GBL09	20	19	129–177	0.85	0.965	0.03052*
	(TGGTG) ₄	20	7	445–575	0.40 (0.80)	0.741 (0.815)	0.00438* (0.76226)
all	all	100	16 ^A	75–620	0.749 ^B	0.9079 ^B	–
Lanyu (Taiwan)	GBL01	27	14	153–193	0.259	0.921	<0.0001*
	GBL02	27	24	104–202	0.680	0.961	0.00024*
	GBL03	27	6	80–140	0.210	0.711	<0.0001*
	GBL07	27	16	114–174	0.684	0.943	0.00576*
	GBL08	27	17	140–186	0.222	0.935	<0.0001*
	GBL09	27	26	136–294	0.851	0.851	<0.0001*
	all	27	16.5 ^A	80–294	0.484	0.887	–

^AMedian number of alleles.

^BAverage.

Table S5. Exact *P*-values of linkage disequilibrium between the seven loci for the five sample sites of *Birgus latro* as calculated using Arlequin, ver. 3.5.1.3 (Excoffier *et al.* 2005)

P-values for the sample sites ECT, HS and TD are given above the diagonal, *P*-values for the sample sites DB and PH below the diagonal. Significant *P*-values ($\alpha = 0.05$) are in bold

Sample site	Locus	GBL01	GBL02	GBL03	GBL07	GBL08	GBL09	(TGGTG) ₄	
ECT & DB	GBL01	—	0.63447	0.18521	0.02131	0.94417	0.56308	0.40904	
	GBL02	0.14686	—	0.56550	0.32604	0.53525	0.03461	0.79073	
	GBL03	0.13212	0.12065	—	0.38462	0.22638	0.54431	0.01626	
	GBL07	0.51592	0.46699	0.26026	—	0.04575	0.19736	0.23009	
	GBL08	0.16263	0.09987	0.81735	0.01724	—	0.28945	0.08673	
	GBL09	0.05233	0.11140	0.11780	0.00318	0.85392	—	0.76884	
	(TGGTG) ₄	0.16980	0.05146	0.26448	0.39033	0.21620	0.54281	—	
	HS & PH	GBL01	—	0.09191	0.15004	0.03368	0.11343	0.46782	0.54822
		GBL02	0.43974	—	0.07250	0.75071	0.14967	0.56429	0.80341
GBL03		0.30806	0.24774	—	0.16858	0.13272	0.46297	0.27968	
GBL07		0.01468	0.73008	0.05973	—	0.17136	0.06541	0.67025	
GBL08		0.59926	0.59442	0.18317	0.52697	—	0.00336	0.17683	
GBL09		0.00673	0.22053	0.62115	0.30104	0.58570	—	0.54494	
TD	(TGGTG) ₄	0.59650	0.88720	0.75438	0.23812	0.29448	0.72000	—	
	GBL01	—	0.26347	0.19964	0.23158	0.62467	0.78748	0.60847	
	GBL02		—	0.56170	0.34511	0.60753	0.80670	0.41120	
	GBL03			—	0.78151	0.63061	0.21252	0.24823	
	GBL07				—	0.02105	0.16947	0.35562	
	GBL08					—	0.24441	0.23665	
	GBL09						—	0.40602	
	(TGGTG) ₄							—	

Table S6. Standard measures of genetic diversity for female and male *Birgus latro* for the four sample sites ECT, HS, PH and TD calculated with GenAIEx, ver. 6.501 (Peakall and Smouse 2012)

n , number of samples; N_a , average number of alleles referring to a sample size of $n = 8$; N_e , average number of effective alleles; H_o , average observed heterozygosity; H_e , average expected heterozygosity; uH_e , average unbiased expected heterozygosity; F_{IS} , inbreeding coefficient; A_p , number of private alleles; s.e., standard error

Sex	Sample site	n	N_a (s.e.)	N_e (s.e.)	H_o (s.e.)	H_e (s.e.)	uH_e (s.e.)	F_{IS} (s.e.)	A_p
Females	ECT	12	10.006 (0.551)	9.178 (0.999)	0.786 (0.040)	0.882 (0.014)	0.921 (0.015)	0.112 (0.037)	17
	HS	10	9.431 (0.483)	7.766 (0.564)	0.771 (0.052)	0.876 (0.010)	0.913 (0.010)	0.111 (0.057)	10
	PH	9	9.842 (0.747)	8.004 (0.955)	0.762 (0.074)	0.863 (0.017)	0.914 (0.018)	0.124 (0.075)	8
	TD	8	9.429 (0.782)	7.567 (0.703)	0.750 (0.055)	0.858 (0.018)	0.915 (0.019)	0.127 (0.059)	6
	all	39	9.972 (0.584)	8.129 (0.407)	0.767 (0.027)	0.868 (0.007)	0.916 (0.007)	0.118 (0.027)	41
Males	ECT	8	10.143 (0.459)	7.997 (0.506)	0.768 (0.079)	0.872 (0.009)	0.930 (0.009)	0.123 (0.086)	7
	HS	10	9.343 (0.709)	7.710 (0.955)	0.671 (0.081)	0.856 (0.020)	0.901 (0.021)	0.223 (0.086)	12
	PH	11	9.424 (0.911)	7.678 (1.151)	0.766 (0.065)	0.848 (0.026)	0.888 (0.027)	0.098 (0.065)	7
	TD	12	10.327 (1.000)	9.786 (1.634)	0.762 (0.086)	0.860 (0.040)	0.898 (0.042)	0.132 (0.083)	14
	all	41	9.823 (0.711)	8.293 (0.561)	0.742 (0.038)	0.859 (0.012)	0.904 (0.013)	0.144 (0.039)	40

Table S7. P-values of the Kruskal–Wallis and significant pairwise Wilcoxon rank-sum tests with Bonferroni correction for comparing the dimensions of the measured morphometric parameters and weight among the four sample sites ECT, HS, PH and TD in female *Birgus latro*

Morphometric parameters: CL, carapace length; CSL, cephalic shield length; TL, thoracic length; TW, thoracic width; W, weight; LCPL, left cheliped propodus length; RCPL, right cheliped propodus length; LCPW, left cheliped propodus width; RDPW, right cheliped propodus width; LCDL, left cheliped dactylus length; RCDL, right cheliped dactylus length; LCML, left cheliped merus length; RCML, right cheliped merus length; AI_CPL, asymmetry index for cheliped propodus length; AI_CPW, asymmetry index for cheliped propodus width; AI_CDL, asymmetry index for cheliped dactylus length; AI_CML, asymmetry index for cheliped merus length; Sample sites: ECT, Eastern Circuit Track; HS, Hosnie’s Springs; PH, Pink House; TD, The Dales

Morphometric parameter (and W = weight)	Kruskal–Wallis P-value	Wilcoxon rank-sum test significant sample site pairs (P-value)
CL	0.0083	HS:TD (0.04)
CSL	0.0874	–
TL	<0.001	ECT:HS (0.0041) HS:TD (0.0097)
TW	0.0041	HS:TD (0.0052)
LCPL	0.1195	–
RCPL	0.0572	–
LCPW	0.3946	–
RCPW	0.3411	–
LCDL	0.4359	–
RCDL	0.1926	–
LCML	0.0394	No significance after Bonferroni correction
RCML	0.2058	–
AI_CPL	0.4591	–
AI_CPW	0.4194	–
AI_CDL	0.0016	ECT:TD (0.0082); HS:TD (0.0175); PH:TD (0.0272)
AI_CML	0.7509	–
W	<0.001	ECT:HS (0.0083); HS:TD (0.0082)

Table S8. P-values of the Kruskal–Wallis and significant pairwise Wilcoxon rank-sum tests with Bonferroni correction for comparing the dimensions of the measured morphometric parameters and weight among the five sample sites ECT, DB, HS, PH and TD in male *Birgus latro*

Morphometric parameters: CL, carapace length; CSL, cephalic shield length; TL, thoracic length; TW, thoracic width; W, weight; LCPL, left cheliped propodus length; RCPL, right cheliped propodus length; LCPW, left cheliped propodus width; RDPW, right cheliped propodus width; LCDL, left cheliped dactylus length; RCDL, right cheliped dactylus length; LCML, left cheliped merus length; RCML, right cheliped merus length; AI_CPL, asymmetry index for cheliped propodus length; AI_CPW, asymmetry index for cheliped propodus width; AI_CDL, asymmetry index for cheliped dactylus length; AI_CML, asymmetry index for cheliped merus length; Sample sites: ECT, Eastern Circuit Track; HS, Hosnie’s Springs; DB, Dolly Beach; PH, Pink House; TD, The Dales

Morphometric parameter (and W = weight)	Kruskal–Wallis P-value	Wilcoxon rank-sum test pairs (P-value)	Significant sample site
CL	0.2642	–	
CSL	0.1444	–	
TL	0.3335	–	
TW	0.7661	–	
LCPL	0.4007	–	
RCPL	0.8325	–	
LCPW	0.4259	–	
RCPW	0.7093	–	
LCDL	0.6003	–	
RCDL	0.3395	–	
LCML	0.2072	–	
RCML	0.3182	–	
AI_CPL	0.0732	–	
AI_CPW	0.3187	–	
AI_CDL	0.2107	–	
AI_CML	0.1773	–	
W	0.3813	–	

Table S9. Estimates of the effective population size N_e for *Birgus latro*

CI, confidence interval

Method	Lowest allele frequency used	N_e	95% CI	
			lower CI	upper CI
Linkage disequilibrium	0.05	492.2	103.7	infinite
	0.02	618.4	257.2	infinite
	0.01	933.6	383.3	infinite
	0+	1595.7	360.5	infinite
Heterozygote-excess	0.05, 0.02, 0.01, 0+	infinite	infinite	infinite
Molecular co-ancestry	0+	690.7	0.7	3467.4

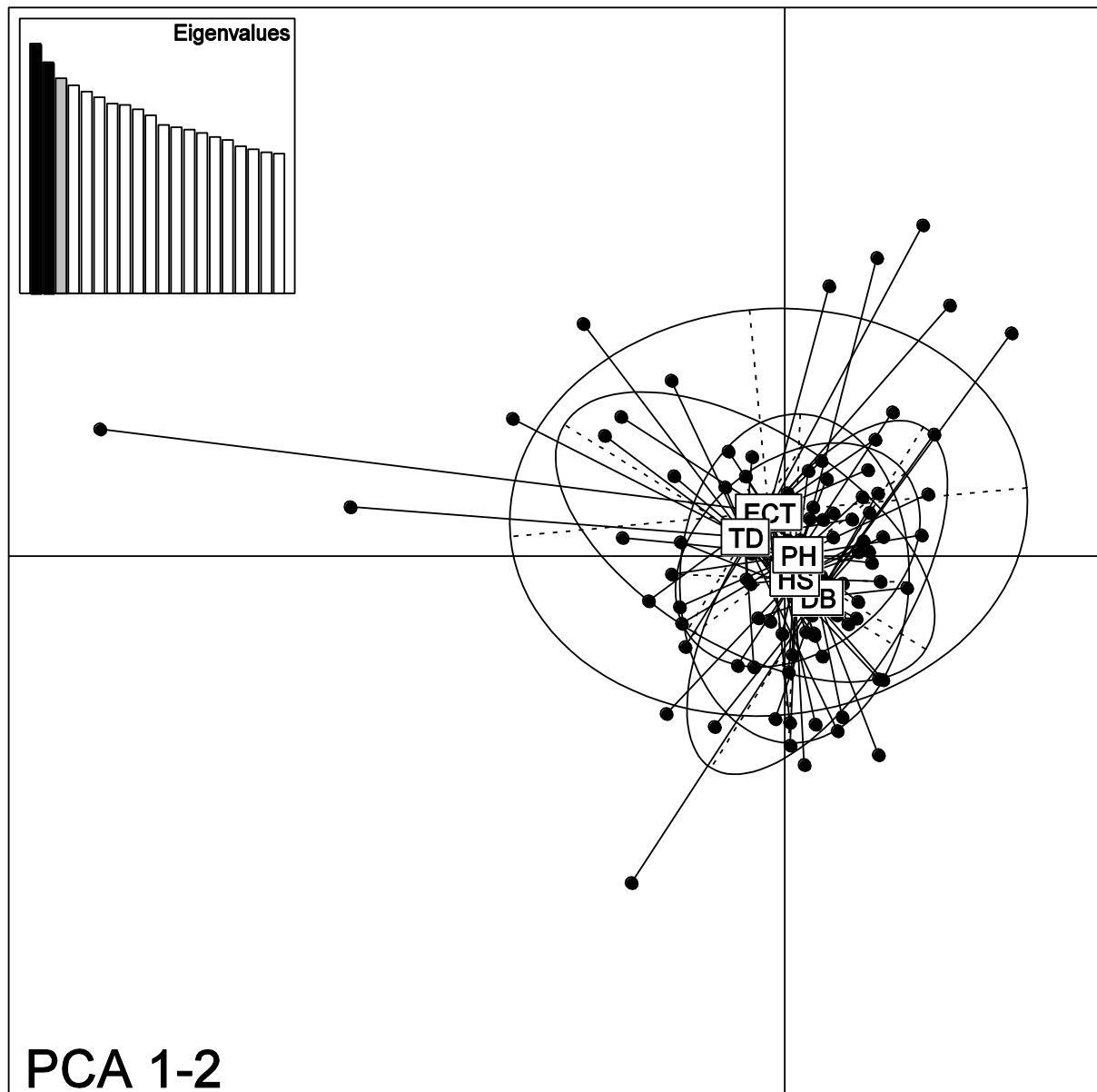


Fig. S1. First and second components (respectively explaining 3.1 and 2.9% of the variability) of a centred and scaled principal component analysis (PCA) of seven-locus microsatellite genotypes of 100 individuals of *Birgus latro* from the five different sample sites ECT, DB, HS, PH and TD. Ovals represent 95% inertia ellipses.

References

- Excoffier, L., Laval, G., and Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetic data analysis. *Evolutionary Bioinformatics Online* **1**, 47–50.
- Gan, C.-H., Tee, S.-M., Tang, P.-C., Yang, J. M.-C., Freire, F., McGowan, A., Narriman, J., Mohammed, M. S., Hsieh, H.-L., Chen, C.-P., Sheppard, C., and Chen, C. A. (2008). Isolation and characteristics of 10 microsatellite markers from the endangered coconut crab *Birgus latro*. *Molecular Ecology Resources* **8**, 1448–1450. doi:10.1111/j.1755-0998.2008.02330.x
- Guo, S., and Thompson, E. (1992). Performing the exact test of Hardy–Weinberg proportion for multiple alleles. *Biometrics* **48**, 361–372. doi:10.2307/2532296
- Peakall, R., and Smouse, P. E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics* **28**, 2537–2539. doi:10.1093/bioinformatics/bts460