

Short Note

Introgressive hybridization between the endangered native *Bombina pachypus* and the introduced *B. variegata* in a protected area in central Italy

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Supplementary material

Information S1.

Individual genotypes

Genotypes of 48 *Bombina* individuals (sample ID) typed in this study for the mitochondrial cytochrome (cytb), the recombination-activating gene 1 (RAG-1) and nine microsatellite loci. Note that codes of cytb haplotypes and RAG-1 alleles agree with those in previous studies (Hofman et al., 2007; Fijarczyk et al., 2011). Sequences of three new cytb haplotypes found in this study (i.e. BW58, BW59, BW60) have been deposited in GenBank (accessions: MN914092-MN914094). County and approximated geographic coordinates (datum WGS84) of sampling localities (pop ID) are given; asterisks indicate tadpoles.

species	sample ID	country	pop ID	lat°(N)/ lon°(E)	cytb	RAG-1	8A	5F	F22	1A	9H	F2	10F	B14	12F
hybrid	LIC_001	Italy	LIC	42.1/12.9	BW21	R3/R3	273/273	146/262	141/141	344/352	169/251	498/498	197/197	175/183	231/231
hybrid	LIC_002	Italy	LIC	42.1/12.9	BW21	R3/R14	271/273	262/266	141/143	344/372	196/251	498/498	197/201	175/183	231/231
hybrid	LIC_003	Italy	LIC	42.1/12.9	BW21	R3/R3	271/271	142/146	141/141	344/352	169/251	498/524	197/197	175/183	227/231
hybrid	LIC_004	Italy	LIC	42.1/12.9	BW21	R3/R3	271/273	142/262	141/141	344/344	251/251	498/498	197/197	175/183	231/231
hybrid	LIC_005	Italy	LIC	42.1/12.9	BW21	R6/R14	271/273	142/146	141/141	344/372	196/251	498/498	197/197	175/175	227/231
hybrid	LIC_006	Italy	LIC	42.1/12.9	A3	R3/R14	271/277	262/266	143/143	336/372	196/239	498/508	197/201	175/183	227/544
hybrid	LIC_007	Italy	LIC	42.1/12.9	BW21	R6/R14	271/271	142/146	141/141	344/372	196/251	498/498	197/197	175/183	231/231
hybrid	LIC_008	Italy	LIC	42.1/12.9	BW21	R3/R3	271/273	142/146	141/141	352/372	196/196	498/498	197/197	175/175	231/231
hybrid	LIC_009	Italy	LIC	42.1/12.9	BW21	R3/R14	271/273	262/262	141/141	352/352	169/196	498/498	197/197	183/183	227/231
hybrid	LIC_010	Italy	LIC	42.1/12.9	A3	R3/R14	271/277	262/266	143/143	336/372	196/239	498/508	197/201	175/183	227/544

hybrid	LIC_011	Italy	LIC	42.1/12.9	BW21	R3/R3	271/273	142/146	141/141	352/372	196/196	498/498	197/197	175/175	231/544
hybrid	LIC_012	Italy	LIC	42.1/12.9	BW21	R3/R14	273/277	146/262	141/143	352/372	169/196	498/498	197/201	175/183	227/231
hybrid	LIC_013	Italy	LIC	42.1/12.9	BW21	R3/R14	271/273	262/262	141/141	352/352	169/196	498/498	197/197	183/183	227/231
hybrid	LIC_014*	Italy	LIC	42.1/12.9	BW21	R3/R3	271/271	142/142	141/141	344/352	169/196	498/498	197/197	183/183	231/231
hybrid	LIC_015*	Italy	LIC	42.1/12.9	BW21	R3/R14	271/277	142/146	141/143	352/372	196/251	498/498	197/201	175/183	231/231
hybrid	LIC_016*	Italy	LIC	42.1/12.9	BW21	R3/R14	273/277	146/262	143/143	352/372	169/239	498/498	197/201	175/183	231/544
hybrid	LIC_017*	Italy	LIC	42.1/12.9	BW21	R14/R14	271/277	146/262	141/143	344/352	196/196	498/498	197/201	183/183	227/544
hybrid	LIC_018	Italy	LIC	42.1/12.9	BW21	R3/R3	271/271	146/266	141/141	344/344	169/251	498/524	197/197	183/183	231/231
hybrid	LIC_019	Italy	LIC	42.1/12.9	BW21	R3/R14	273/277	266/266	143/143	336/344	196/251	498/498	197/197	175/183	544/544
hybrid	LIC_020	Italy	LIC	42.1/12.9	BW21	R3/R14	271/271	146/262	141/141	344/372	196/251	498/498	197/197	175/175	231/231
hybrid	LIC_021	Italy	LIC	42.1/12.9	BW21	R3/R14	271/273	146/266	141/141	352/372	169/239	498/508	197/201	175/183	227/231
<i>B. pachypus</i>	FAV_001	Italy	pop_21	42.1/12.8	A3	R14/R14	277/277	266/270	143/143	344/348	243/255	508/508	197/201	175/175	544/544
<i>B. pachypus</i>	FAV_002	Italy	pop_21	42.1/12.8	A3	R14/R14	273/277	270/270	143/143	352/352	243/243	508/508	197/205	175/175	544/544
<i>B. variegata</i>	PUK_001	Albania	PUK	42.0/19.9	BW60	na	302/318	124/140	137/137	344/348	153/165	468/514	197/201	185/185	215/215
<i>B. variegata</i>	PUK_002	Albania	PUK	42.0/19.9	BW59	R1/R17	271/314	136/140	141/141	348/368	161/224	470/560	197/209	185/195	219/227
<i>B. variegata</i>	PUK_003	Albania	PUK	42.0/19.9	BW53	R12/R17	271/271	132/144	141/141	344/348	175/201	468/480	197/209	181/183	215/227
<i>B. variegata</i>	PUK_004	Albania	PUK	42.0/19.9	BW21	R1/R17	271/314	124/140	147/147	348/348	213/217	476/484	189/197	181/195	215/227
<i>B. variegata</i>	PUK_005	Albania	PUK	42.0/19.9	BW59	R7/R17	271/306	132/144	141/147	348/348	165/224	470/560	189/209	189/195	215/227
<i>B. variegata</i>	PUK_006	Albania	PUK	42.0/19.9	BW58	R6/R7	271/338	136/150	145/145	352/352	241/253	468/484	201/205	183/185	215/215
<i>B. variegata</i>	PUK_007	Albania	PUK	42.0/19.9	BW60	R3/R12	271/338	124/140	133/133	352/368	187/187	468/488	197/209	185/195	227/231
<i>B. variegata</i>	PUK_008	Albania	PUK	42.0/19.9	BW58	R6/R17	271/314	136/140	137/137	352/352	241/253	468/480	189/205	181/185	215/215
<i>B. variegata</i>	BES_001	Italy	BES	46.0/11.2	BW8	R21/R21	320/320	142/142	141/141	320/320	153/157	484/484	209/213	165/171	227/227
<i>B. variegata</i>	GAI_001	Italy	GAI	45.7/10.6	BW8	R21/R21	na	na	141/141	na	157/157	484/484	205/205	165/165	219/227
<i>B. variegata</i>	GAI_002	Italy	GAI	45.7/10.6	BW8	R21/R21	320/320	na	141/141	na	157/157	na	205/205	165/165	219/227
<i>B. variegata</i>	GAI_003	Italy	GAI	45.7/10.6	BW8	R21/R21	320/320	146/146	141/141	na	157/157	486/486	205/205	165/165	227/227
<i>B. variegata</i>	GAI_004	Italy	GAI	45.7/10.6	BW8	R21/R21	320/320	na	141/141	na	157/157	na	205/205	165/165	227/227
<i>B. variegata</i>	GAI_005	Italy	GAI	45.7/10.6	BW8	R21/R21	320/320	146/146	141/141	320/320	157/157	484/486	205/205	165/165	227/227
<i>B. variegata</i>	GAI_006	Italy	GAI	45.7/10.6	BW8	R21/R21	320/320	146/146	141/141	320/320	157/157	484/486	205/205	165/165	219/227
<i>B. variegata</i>	LOL_001	Italy	LOL	46.2/11.2	BW8	R21/R21	320/324	112/112	141/141	320/320	157/169	na	na	165/167	227/227
<i>B. variegata</i>	LOP_001	Italy	LOP	45.9/10.9	BW8	R21/R21	324/324	112/112	141/145	320/320	153/157	476/484	205/221	165/171	231/236
<i>B. variegata</i>	MBA_001	Italy	MBA	45.8/10.9	BW8	R21/R21	287/316	112/112	141/141	320/320	169/169	476/476	205/213	165/165	219/231
<i>B. variegata</i>	MLO_001	Italy	MLO	46.2/11.1	BW8	R21/R21	287/320	112/142	141/141	320/320	161/169	484/484	217/217	165/165	215/219

<i>B. variegata</i>	NAG_001	Italy	NAG	45.9/10.9	BW8	R21/R21	320/324	142/142	141/141	320/320	157/157	484/486	205/205	165/165	219/231
<i>B. variegata</i>	NAG_002	Italy	NAG	45.9/10.9	BW8	R21/R21	320/320	142/142	145/145	320/320	157/169	476/484	205/205	165/165	219/227
<i>B. variegata</i>	POL_001	Italy	POL	45.8/10.9	BW8	R21/R21	324/324	112/112	141/141	320/320	157/157	476/484	217/221	165/171	223/231
<i>B. variegata</i>	SEG_001	Italy	SEG	46.2/11.3	BW8	R21/R21	287/324	112/112	145/145	320/320	157/157	476/490	209/213	169/171	215/223
<i>B. variegata</i>	VER_001	Italy	VER	46.2/11.3	BW8	R21/R21	320/324	112/142	141/145	320/320	165/169	484/484	na	167/171	na
<i>B. variegata</i>	ZAV_001	Italy	ZAV	46.2/11.1	BW8	R21/R21	287/320	112/142	141/141	320/320	153/169	484/484	213/217	165/171	219/231

Information S2.

Procedures for amplification and genotyping of *cytb*, RAG-1 and microsatellite loci

We genetically characterised *Bombina* samples using a fragment of the nuclear recombination activating gene 1 (RAG-1, 1061 bp) and mitochondrial cytochrome b gene (*cytb*, 1096 bp), and nine microsatellite (*msat*) loci (see table below). PCR reactions were performed in a total volume of 12 μ L containing 20-60 ng of genomic DNA, 2X PCR Master Mix (Thermo Fisher Scientific) and 0.51 μ M each primer. Amplifications were carried out in a 2720 Thermal cycler (Applied Biosystems) as follows: initial denaturation at 94°C for 4', followed by 35 cycles at 94°C for 30", a locus-specific annealing temperature (see table below) for 45", 72°C for 45", and a final elongation step at 72°C for 7'. Amplicons were electrophoresed on a 1-1.5 % agarose gel with ethidium bromide and visualized under UV light to assess successful reactions.

Fragment analysis of microsatellites was conducted on a 3730xl 96-capillary genetic analyzer Applied Biosystems, Thermo Fisher Scientific) by the DNA Analysis Facility on Science Hill (Yale University, USA), and we determined genotypes using Peak Scanner v.2 (Applied Biosystems). PCR products of *cytb* and RAG-1 were Sanger sequenced (Big Dye terminators) with an ABI3730xl automated DNA sequencer Applied Biosystems) by the facility mentioned above. The chromatograms were assembled and edited in CodonCode Aligner v 7.1.2 (CodonCode Corporation). RAG-1 alleles were phased in DnaSP 6.1 (Rozas et al., 2017) using the no-recombination model implemented in PHASE 2.1 (Stephens and Donnelly, 2003).

Marker	Primer	Primer sequence (5'--> 3')	Annealing (T°)	Reference
RAG-1	Rag1L	GAGGAAAGCCCTATACTTCTGG	55	Fijarczyk et al. 2011
	Rag1H	CTCCATGATGGCCTCCAAT		
<i>cytb</i>	L16245	CCTTAATGGCACCCAACCT	55	Hofman and Szymura 2007
	H17444	TGAACCTTCACTCTTCGGTTT		

msat	BobomF2.for	AGCAGAGATGAGAGGACAGTG	55	Stuckas and Tiedemann 2006
	BobomF2.rev	TCAGGGGTAGCAGATTTTCAG		
msat	BobomB14.for	ACTAACCTGCCACATAACTTG	55	Stuckas and Tiedemann 2006
	BobomB14.rev	CTGGGTTTTTTAATTGGAAGG		
msat	BobomF22.for	AGGCAAAGGATTCTGAGAATG	48	Stuckas and Tiedemann 2006
	BobomF22.rev	CCTCAAAGTCGAAAAATATT		
msat	Bobom8A.for	AATTTCTTAGTGCTGCCAATTGC	55	Hauswaldt et al. 2007
	Bobom8A.rev	GGGAAGGGACATTTTAGCTACATAC		
msat	Bobom9H.for	AACAGCCATTATTTAAACCATTAG	48	Hauswaldt et al. 2007
	Bobom9H.rev	CAATAAAGCAGTATTTCCCAAATG		
msat	Bobom12F.for	ATAGGAGGTTTATAATGAAAGGGCAAC	55	Hauswaldt et al. 2007
	Bobom12F.rev	GATTGGATTGGGCTATGATATTCTG		
msat	Bobom1A.for	ATGTGGCTTCCATTGACCTTTCG	55	Hauswaldt et al. 2007
	Bobom1A.rev	CATGCCAAGAAGGATTGAGTCTGTC		
msat	Bobom5F.for	ATGAATTGGAAGGTAAGAACTTACACC	55	Hauswaldt et al. 2007
	Bobom5F.rev	CAAATGATACAAATCAAGTGAATGG		
msat	Bobom10F.for	ATCCAACTTCAAATTCACAGGTCAC	55	Hauswaldt et al. 2007
	Bobom10F.rev	ACAAGGGATACCAGGAGAACAAAGC		

Information S3.

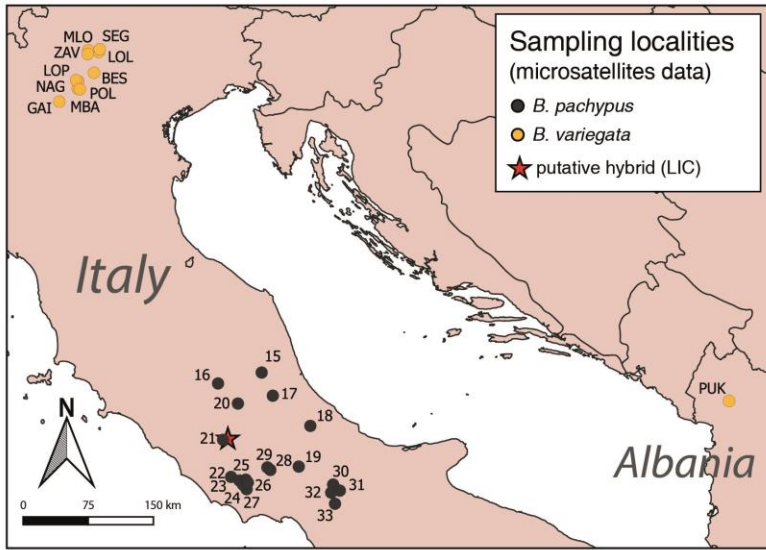
Validation of reference samples

A preliminary clustering analysis in STRUCTURE v.2.3.4 (Pritchard, Stephens, and Donnelly, 2000) was run to validate the reference groups, i.e. *B. pachypus* (149 samples from 19 localities; data from Talarico et al., 2019) and *B. variegata* (25 samples from 12 localities) (fig. A). Analysis settings were as follow: admixture model with correlated allele frequencies; five repeated runs for each K value in the range 1-5; 500,000 iterations after a burn-in period of 200,000. The most probable number of K was inferred choosing the highest ΔK value (Evanno, Regnaut and Goudet, 2005) in STRUCTURE HARVESTER (Earl and vonHoldt, 2012). Finally, we combined the results of multiple runs for the chosen optimal K in CLUMPAK (Kopelman et al., 2015).

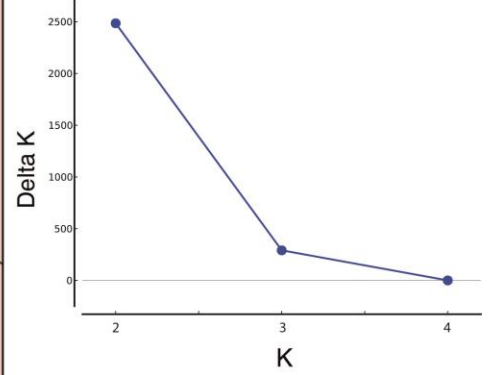
Two genetic clusters were supported according to Evanno's method (fig. B). Clusters unambiguously corresponded to the two species (fig. C).

Figures A-C. (A) Map of sampling localities for *B. pachypus* and *B. variegata* reference samples. Population IDs refer to supplementary text S1 and Talarico et al. (2019); (B) The optimal number of clusters according to the Evanno's method. The maximum ΔK value was observed when $K = 2$; (C) Bar plot of admixture analysis (STRUCTURE) for $K = 2$.

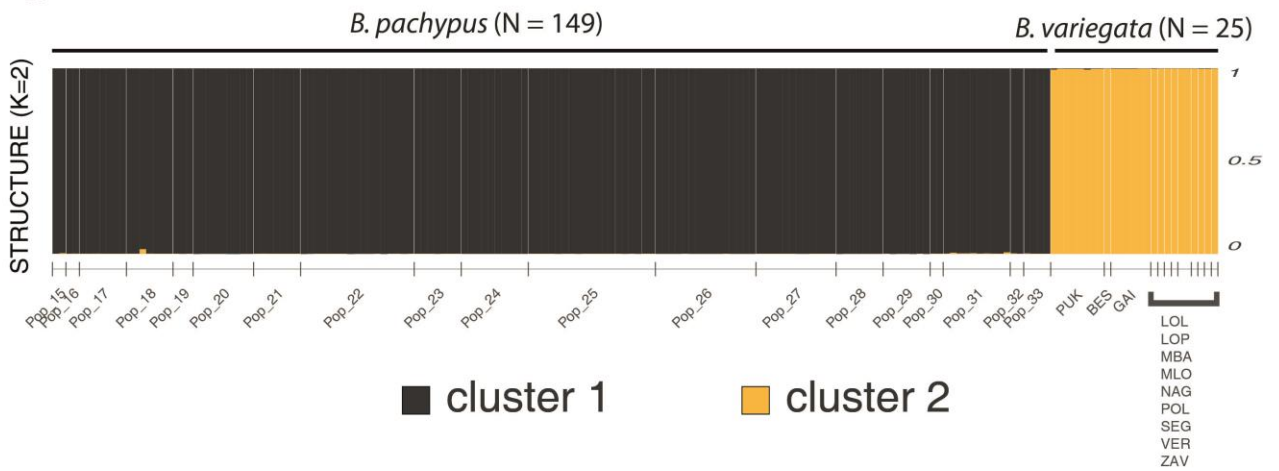
A



B



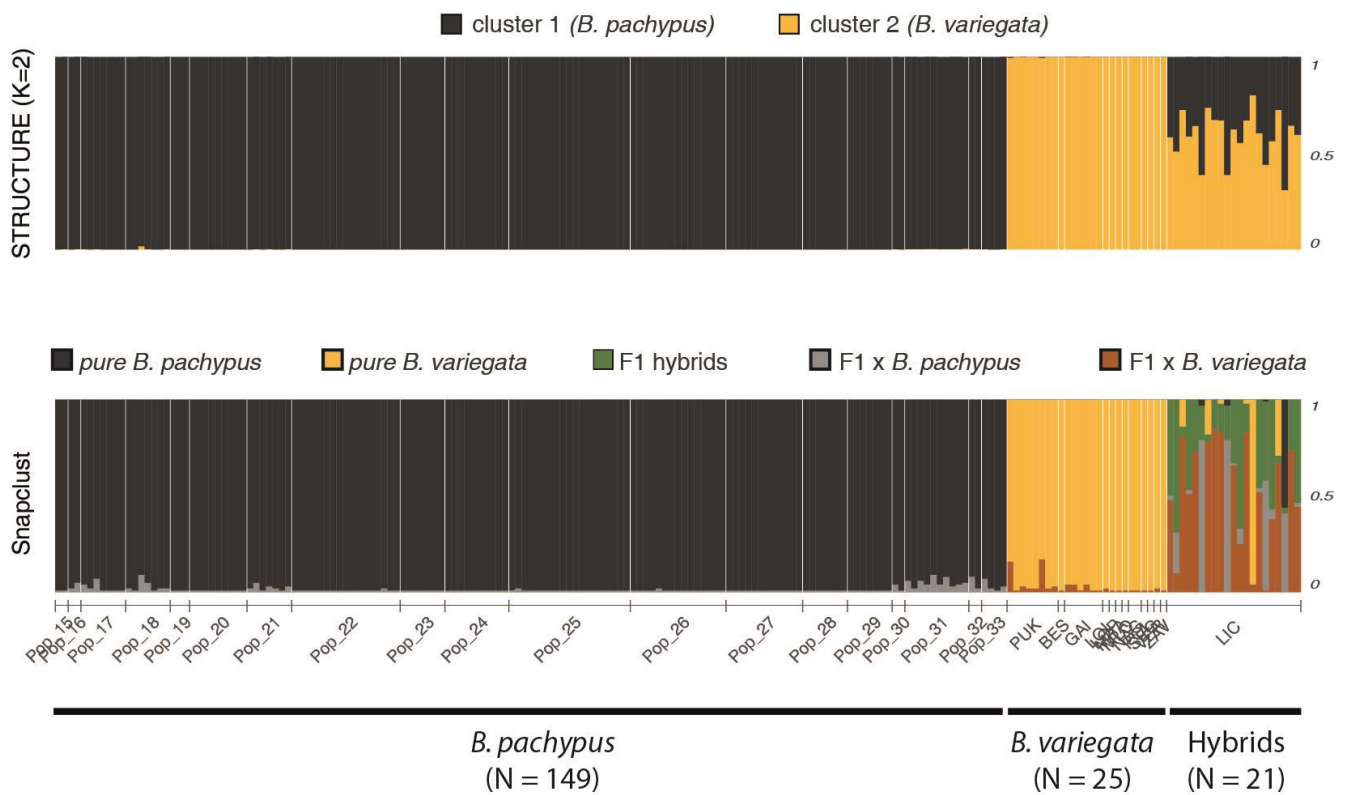
C



Information S4.

Results of STRUCTURE and Snapclust analyses

Admixture proportions (upper panel: USEPOPINFO model in STRUCTURE v.2.3.4; Pritchard, et al., 2000) and assignment probabilities (lower panel: Snapclust function in adegenet; Beuguin et al., 2018) for overall 195 *Bombina* (9 microsatellite loci).



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