Interspecific hybridization of *Quasipaa* and genetic characteristics of hybrid tadpole

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This study aims to reveal the formation mechanism of distant hybridization of *Quasipaa*. We collected five species of *Quasipaa* for hybridization experiment and raised tadpoles at three temperature groups (14 ℃, 22 ℃, and 28 ℃) and three density groups (5, 15, and 30 ind/L). We monitored the growth rate and swimming speed of the tadpoles. We also used nine microsatellite markers to evaluate genetic diversity and structure between the crossbred offspring and parents. Results suggested that the hybrid combinations of *Quasipaa spinosa* (♀) × *Q. shini* (♂) and *Q. boulengeri* (♀) × *Q. spinosa* (♂) obtained healthy crossbred offspring. Temperature and breeding density significantly affected the growth and development of purebred and crossbred tadpoles. Compared with purebred tadpoles, the hybrids showed heterosis under similar experimental conditions. The genetic diversity of the crossbred tadpoles was higher than that of the parents. Higher heterozygosity and genetic differentiation were also observed in the progeny population. A close genetic relationship was found between the offspring population and the female parent.

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Abstract: This study aims to reveal the formation mechanism of distant hybridization of *Quasipaa*. We collected five species of *Quasipaa* for hybridization experiment and raised tadpoles at three temperature groups (14 ℃, 22 ℃, and 28 ℃) and three density groups (5, 15, and 30 ind/L). We monitored the growth rate and swimming speed of the tadpoles. We also used nine microsatellite markers to evaluate genetic diversity and structure between the crossbred offspring and parents. Results suggested that the hybrid combinations of *Quasipaa spinosa* (♀) × *Q. shini* (♂) and *Q. boulengeri* (♀) × *Q. spinosa* (♂) obtained healthy crossbred offspring. Temperature and breeding density significantly affected the growth and development of purebred and crossbred tadpoles. Compared with purebred tadpoles, the hybrids showed heterosis under similar experimental conditions. The genetic diversity of the crossbred tadpoles was higher than that of the parents. Higher heterozygosity and genetic differentiation were also observed in the progeny population. A close genetic relationship was found between the offspring population and the female parent.

Key words: *Quasipaa*; hybridization; heterosis; genetic diversity

1 Introduction

Distant hybridization between species, intergeneric, and higher taxonomic species can integrate the genome of the parents into the hybrid progeny to produce rich genetic variation (Zhang et al., 2014). Distant hybridization plays an important role in adaptation (Song et al., 2011), extinction (Vonlanthen et al., 2012), and differentiation of species (Yakimowski et al., 2014). Early studies on amphibian distant hybridization focused on hybridization experiments (Gherghel et al., 2012; Francillonvieillot et al., 1990; Mikulíček et al., 2008; Parris et al., 1999; Sherman et al., 2010; Gray et al., 2011; Picker et al., 1985). Ding (1956) conducted a hybrid experiment between *Pelophylax nigromaculatus* and *P. plancyi* and found that both frogs can obtain normal hybrid seed generation. Many new modern technologies have been applied to amphibian hybridization research. Such technologies use chromosomes (Pereyra et al., 2009), sounds (Guerra et al., 2011; Lemmon et al., 2010; Roberts et al., 2010), allozyme sites (Yanchukov et al., 2006), mitochondrial genes (Correa et al., 2012), and nuclear genes (Hauswaldt et al., 2011; Simões et al., 2012).

Quasipaa (Anura: Dicroglossidae) is a unique genus in Asia and one of the most widely used frog resources in China (Fei et al., 2005). This genus is known for keratinized skin-spines on the chest. In recent years, the natural populations of this genus have declined or even gone extinct because of overharvesting and exploitation for human consumption, water pollution, and habitat destruction and degradation (Wang et al., 2004). In particular, *Q. spinosa* is captured for a long time because it is edible and has medicinal value (Shu, 2000; Zheng et al, 2010). Our preliminary study used mitochondrial genes and nuclear genes as gene markers and identified different

degrees of introgression hybridization in *Quasipaa* species (Zhang et al., 2018). A wide distribution of overlapping regions and amplexus error exist during the breeding season in *Quasipaa* species (Yu et al., 2008). In this regard, scholars have speculated the presence of widespread hybridization phenomenon in *Quasipaa* species in the sympatric distribution region. Such phenomenon leads to the differentiation of new species. In this study, five species of *Quasipaa* were selected for orthogonal and reverse experiments and molecular biology tests for the following: 1) detect whether the interspecific hybridization of *Quasipaa* can produce offspring; 2) determine the fitness of the hybrid offspring; and 3) evaluate the genetic characteristics of the hybrid progeny. Results not only provide strong evidence for the hybridization of amphibians but also provide theoretical support for the formation and assessment of species and biodiversity and for breeding of *Quasipaa*.

2 Materials and Methods

2.1 Sampling of specimens

A total of 309 individuals of five species of *Quasipaa* were obtained from six overlapping regions across South China from 2015 to 2016 (Table 1). All frogs were initially identified based on morphological traits and by molecular methods and then labeled using haplotype numbers. Dead frogs were frozen at −80 °C, and surviving frogs were fed for subsequent DNA sequencing and hybridization.

2.2 Orthogonal and reverse experiments

Eight combinations of *Q. spinosa* and *Q. shini*, *Q. boulengeri*, *Q. jiulongensis,* and *Q. exilispinosa* were selected on the basis of the molecular biology results to obtain 40 groups for

hybridization experiments in 2015-2016. The samples were grouped in a 1:1 ratio (self-made imitation ecological frog pool) with the following parameters: stocking density of 6-10 ind/m^2, water temperature of 20 °C-25 °C, pH of 6.5–8.0, dissolved oxygen of 6.5 mg/L, and bait fed once daily. A one-time injection of chorionic gonadotropin (HCG, 5000 units/support) and luteinizing hormone releasing hormone A3 (LRH-A3, 25 μg/support) (all produced in Ningbo City, Zhejiang Province, the second hormone factory) into leg muscles was conducted for artificial oxytocin reproduction. The dose for male frogs was 1/2 of the dose for female frogs. The injected frog was placed in the spawning pool and observed once every 12 hours.

2.3 Water temperature and density experiments

We selected healthy hybrid tadpoles of Jinhua *Q. spinosa* (♀) and Lushan *Q. shini* (♂) and purebred tadpoles of *Q. spinosa* hatched on the same day for experiment. All experimental samples were first kept at 22 °C for 5 days and stocked into a pre-prepared culture box (40 cm × 30 cm × 40 cm) at temperature, controlled with a chiller and a heating rod. Body length m_1 and body weight w_1 of each tadpole were measured with a caliper (accurate to 0.01 cm) and an electronic balance (accurate to 0.001 g), respectively, before stocking. All samples were raised to metamorphosis at three different temperatures: 14 °C, 22 °C, and 30 °C; and three different densities: 30 ind/L (150), 15 ind/L (75), and 5 ind/L (25). Forty tadpoles were subjected to temperature treatment, where water temperature variation was controlled at ± 2 °C and water depth was 20 ± 2 cm. In density treatment, the water level was set at 5 L and the temperature was maintained at 23 ± 2 °C. Two parallel set ups were processed for each treatment, and one set of

purebred tadpoles was used as control. The tadpoles were exposed to a 12 L: 12 D photoperiod throughout the study period, and water in the containers was changed weekly.

All data were tested by normal distribution and homogeneity of variance before statistical analysis in SPSS 20.0. The significance criterion was set to 0.05. Calculations were conducted using the following equations:

Body size growth rate = m_2-m_1/t_2-t_1; body mass growth rate = w_2-w_1/t_2-t_1; speed = M/t, where m_1 and m_2 represent the body size; w_1 and w_2 represent the body mass; t_1 represents the time of m_1 and w_1, t_2 represents the time of m_2 and w_2; M represents the swimming distance; and t represents the time used.

2.4 DNA extraction, sequencing, and phylogenetic analyses

The DNA genome of 31 parental *Q. spinosa* and 24 *Q. shini* were extracted by noninvasive sampling method using Genomic DNA kits (Sangon) in accordance with the manufacturer's protocol (Zheng et al., 2018). Genomic DNA was extracted from the muscles of 31 hybrid tadpoles by microdamage sampling. The published microsatellite loci of the related species of *Quasipaa* were selected, and nine pairs of the microsatellite primers were screened and amplified (Zheng et al., 2009; Xia et al., 2013; Yuan et al., 2015; Khudamrongsawat et al., 2013). The total microsatellite PCR reaction system was 30 μL containing 20 μL of the mixture of 10 μL of 2× Es TaqMastermix, 0.7 μL of primers, 8 μL of ddH_2O, and 0.6 μL of DNA template. The PCR reaction was conducted as follows: initial denaturation for 5 min at 95 °C; 30 cycles of denaturation for 30 s at 94 °C, annealing (PSP2, PSP3, SSR3, SSR6, and SSR9 were 50 °C;

PSP13 and SSR11 were 53 °C; PSP15 was 55 °C; and SSR4 was 59 °C) for 30 s, extension for 30 s at 72 °C, and final extension at 72 °C for 8 min.

The file format of microsatellite data was converted using Convert software (Glaubitz, 2004). Allele numbers (N_A), effective allele numbers (N_E), observed heterozygosity (*Ho*), and expected heterozygosity (H_E) for each population were determined using Popgene32 software. Polymorphism information content (*PIC*) was calculated using Cervus 3.0 software (Kalinowski et al., 2007). The inbreeding coefficient (F_{IS}) of the population was calculated by Fisher's exact test with Bonferroni's correction using FSTAT1.2.1 software (Goudet, 1995). The genetic differentiation coefficient (F_{ST}) and inter-species analytical variation (AMOVA) of the population were analyzed using Arlequin 3.11 software (Excoffier & Lischer, 2010).

Structure 2.3.3 was utilized to assess the population structure with Bayesian clustering and an admixture model from $K = 2$ to $K = 11$ in 100 runs. Assignment clusters were made with burn-in of 50,000 and 100,000 Markov Chain Monte Carlo iterations. CLUMPP software was used to merge all runs for each K, and the results were visualized by Distruct 1.1. The best K value was estimated using the Structure Harvester (Pritchard et al., 2000) online tool.

Bottlenecks in the case of unknown historical populations were estimated using Bottleneck software as a measure of the population's effective population and genetic rate of change (Cornuet & Luikart, 1996). The Wilcoxon sin-rank test was used to perform the operation using the infinite allelic model (LAM), the two-phase mutation model (TPM), and the stepwise mutation model (SMM) (Spencer et al., 2000).

3 Results

3.1 Fertilization rate and hatching rate of each combination

The fertilization rate of all hybrid combinations was above 80%, except for the two hybrid combinations of *Q. spinosa* (♀) × *Q. jiulongensis* (♂) and *Q. jiulongensis* (♀) × *Q. exilispinosa* (♂). The hatching rate was low. Only the hatching rate of the hybrid group of *Q. spinosa* (♀) × *Q. shini* (♂) was close to 50%, which was slightly lower than that of the *Q. spinosa* self-crossing group. Although the hybrids of *Q. boulengeri* (♀) × *Q. spinose* (♂) had healthy offspring, the hatching rate was extremely low ($P<0.05$) (Table 2).

3.2 Growth rates and swimming speed of purebred and hybrid tadpoles

3.2.1 Different water temperature conditions

The growth and development indices of two tadpoles significantly differed among the three temperature groups. The growth rate of the body length (14 °C: t=-8.061, P=0.001; 22 °C: t=-4.609, P=0.01; 30 °C: t=-2.871, P=0.045) and body weight (14 °C: t=-8.854, P=0.001; 22 °C: t=-6.379, P=0.003; 30 °C: t=1.000, P=0.1) of the hybrids were significantly higher than those of the purebred tadpoles (Figure 1). In the 22 °C group, the speed of the hybrid tadpoles (t=0.519, P=0.001) exceeded that of the purebred tadpoles after 80 days; the other groups were slower than the purebred tadpoles (Figure 2).

3.2.2 Different density conditions

In the 30 ind/L density group, the growth rate of the body length of the hybrid tadpoles (t=-

24.495, $P<0.001$) was significantly greater than that of the purebred tadpoles. The differences in the growth rate of the body length (5 ind/L: t=-2.052, P=0.11; 15 ind/L: t=-2.683, P=0.055) and body weight (5 ind/L: t=2.479, P=0.068; 15 ind/L: t=-2.372, P=0.077; 30 ind/L: t=-2.530, P=0.065) of two tadpoles were not significantly differed among the other treatment groups. As the density increased, the body length growth rate, body weight growth rate, and speed of the hybrid tadpole gradually exceeded those of the purebred tadpoles (Figures 3 and 4).

3.3 Genetic diversity and genetic structure of three populations

Among the three populations, the ranges of the observed alleles and effective alleles were 3.78–8.44 and 2.20–4.48, respectively. The ranges of the observed heterozygosity and expected heterozygosity were 0.38-0.88 and 0.32-0.72, respectively. The average number of alleles in the population was significantly larger than the average number of the effective genes ($P<0.01$). The average expected heterozygosity of the hybrid tadpoles was higher than that of the two parents. The range of the average polymorphic information content value was 0.42-0.59, with an average of 0.51. The inbreeding coefficient of the hybrid tadpoles was negative (Table 3).

The population molecular variance analysis (AMOVA) showed that the molecular variances accounted for 75.92% within populations and 24.08% among populations (Table 4). Hence, genetic variations in the three populations occurred mostly within populations.

The analysis of population bottleneck effect showed that in IAM mode, the hybrid tadpoles showed a significant bottleneck effect ($P<0.01$); in TPM mode, the three populations were in equilibrium and no bottleneck effect was detected; in SMM mode, Jinhua *Q. spinosa* showed a

significant bottleneck effect (P<0.01), and the other populations were in equilibrium (Table 5). Analysis with microsatellite markers indicated higher Fst between the hybrid tadpoles and the two parental populations (Table 6). Structure software was used for clustering individuals into 2 ≤ K ≤ 10. The best K value was 3 by ΔK=m|L″(K)|/s|L(K)| (Figure 5). The three populations were grouped into three clusters, with different degrees of gene flow among the three populations (Figure 6).

4 Discussion

4.1 Feasibility of hybridization among *Quasipaa* species.

In this study, we selected five species of frogs and eight combinations for hybridization experiments. The hybrids of *Q. spinosa* (♀) × *Q. shini* (♂)obtained healthy hybrid offspring. The molecular biology analysis of *Quasipaa* fully verified the hybridization among the species.

The reciprocal combination of *Q. spinosa* (♀) × *Q. shini* (♂) did not produce healthy hybrid offspring. In the amphibious interspecific hybridization, the orthogonal can obtain progeny but the reciprocal cannot; for example, the combination of *Rana Plancyi* (♀) × *Bufo Raddei Strauch* (♂) yielded few small tadpoles, and no cleavage occurred in the reverse crossover (Parris et al., 1999). The hybrid *Q. boulengeri* (♀) × *Q. spinosa* (♂) underwent multiple hybridization experiments and produced few offspring. Further research should be conducted to determine whether the small number of offspring are produced by the combination of sperm from *Q. spinosa* and eggs from *Q. boulengeri* or if the eggs are stimulated by *Q. spinosa* sperm.

189 The hybridization of *Quasipaa* was related to the genetic distance. Based on the genetic
190 distance analysis (Yu et al., 2008), the genetic basis of *Q. spinosa* and *Q. boulengeri* differed
191 because of their distant genetic relationship; thus, the compatibility of chromosomes between the
192 two parents was poor and normal fertilization was difficult to complete. However, the genetic
193 basis of *Q. spinosa* and *Q. shini* was relatively small; hence, the cross between *Q. spinosa* and *Q.*
194 *shini* is more likely to occur.

195 No hybrid generation was found in the cross among *Q. spinosa*, *Q. jiulongensis*, and *Q.*
196 *exilispinosa*, suggesting that this phenomenon was caused by the long genetic distance and huge
197 genetic differences between the both sides.

198 **4.2 Heterosis of hybrid tadpole**

199 Two theories have been established in the study of amphibious natural hybridization zone.
200 First, the hybridization of two species located in the same distribution region will cause the
201 disorder of the parental genotype and reduce the fitness of hybrid offspring (Loftus & Jasper,
202 1975). Second, if a mutual overlapping area exists between two species, then hybridization may
203 occur between the two species, and the hybrid offspring can adapt to the habitat environment of
204 the two parents, showing higher adaptability.

205 Moore (1977) believed that heterosis maintains the existence of many hybrid bands. The two
206 theories suggest that hybridization is a random process. In the present study, the growth and
207 development indices of the hybrid tadpoles in the three temperature treatment groups were
208 superior to those of the purebred tadpoles, showing heterosis; this finding is similar to the results

of *Rana blairi* (Roberts, 2010). The temperature of 22 °C was more suitable for the growth of the two kinds of tadpole, consistent with the metamorphosis of *Q. spinosa* tadpoles (Zhang et al., 2014). When the living environment is fixed, increasing the population density will lead to intense intraspecific competition, thereby affecting the growth rate, metamorphosis time, individual size, and survival rate of the tadpoles (Relyea & Hoverman, 2003). The growth and development indicators of the two tadpoles decreased significantly with increasing density, consistent with previous research results (Hailey et al., 2007). The growth and development indices of the hybrid tadpoles were slightly higher than those of purebred species in the same period because of the higher adaptability of the former in the high-density environment.

Numerous studies have pointed out that species fitness is affected by multiple factors, such as external (such as environment) and internal factors (such as genotype). Fitness is a relative concept and should be considered in relation to the actual growth and development stage. In the present experiment, the growth rate and speed of the hybrids and purebred tadpoles were investigated at different temperatures and densities. Research on species fitness should focus on entire life history and even the entire hybrid lineage. Fitness should be studied from various aspects and perspectives of the fitness of hybrid offspring, such as singing, sexual selection, hibernation, etc. The fertility of hybrid tadpoles should be further studied because of the long growth cycle of *Quasipaa* species.

Hybrids generally do not produce new species, except for fertile offspring that occupy new niches or are no longer backcrossed with their parents; the offspring become more fertile and

adaptable, and new species are produced. For example, *Pleurotus esculentus* is a hybrid progeny species produced by crossing *P. ridibundus* and *P. lessonae* (Arioli et al., 2010). The offspring produced by interspecific hybridization of *Quasipaa* have higher fitness than the purebred generation. This study also provides strong evidence for the hybridization of *Quasipaa*. The results should be further validated by field surveys combined with morphological studies.

4.3 Genetic diversity and structure of three populations

Allele number (N_A) and heterozygosity (*Ho*) are two important parameters used to study the population genetic diversity of organisms. In this study, based on the analysis of microsatellite markers, the three populations had extremely high genetic diversity. The polymorphism information content (*PIC*) was one of the indicators for measuring allelic polymorphism. High *PIC* suggests that the hybrid progeny has higher genetic diversity, which may be the embodiment of the hybridization advantage among species. The inbreeding coefficient of the hybrid tadpoles was negative, suggesting that the progeny population experienced bottleneck and affected the population structure in population formation. In the present study, we analyzed the genetic structure of the population between the offspring and the parents. All of the three populations had higher differentiation levels, and intraspecific variation was the main source of variation of the population. The structure analysis with the Reynolds genetic distance revealed the clustering of the three populations, consistent with the results from the pairwise FST and phylogenetic network. The three populations were consistently separated and became an independent cluster. Gene flow was found among multiple individuals of the progeny population and their parents.

The gene contains a large proportion of maternal gene content, indicating that the hybrid tadpoles are more related to *Q. spinosa*. The different levels of gene flow among the populations further validated the hybrid theory.

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357

Table 1(on next page)

GPS coordinates and sample sizes for each geographic location.

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location	Coordinates		*Q. spinosa*	*Q. exilispinosa*	*Q. boulengeri*	*Q. jiulongensis*	*Q. shini*
Yichun	E114°38′27″	N27°82′05″	29	0	37	0	0
Anhua	E111°31′22″	N28°30′42″	25	0	23	0	0
Jinhua	E119°14′7″	N28°32′23″	36	0	0	0	0
Wuyishan	E118°0′36″	N27°16′12″	28	8	0	21	0
Lushan	E116°13′19″	N29°40′06″	0	0	0	0	24
Longsheng	E109°58′48″	N25°49′12″	30	0	40	0	8
Total			148	8	100	21	32

1

Table 2(on next page)

Fertilization and hatching rate of each cohort in the genus *Quasipaa*

Notes: “JX,” “JF,” “JC,” “JL,” and “XJ” represent *Q. spinose*, *Q. boulengeri*, *Q. shini*, *Q. jiulongensis*, and *Q. exilispinosa*, respectively; different superscript letters in the same column indicate significant differences (P<0.05).

	fertilization rate	hatching rate
JX♀ × JF♂	88.06 ± 3.87[a]	0
JF♀ × JX♂	84.15 ± 5.15[ab]	3.60 ± 2.55[c]
JX♀ × JC♂	87.20 ± 3.88[a]	47.12 ± 5.45[b]
JC♀ × JX♂	84.00 ± 5.66[ab]	0
JX♀ × JL♂	0	0
JL♀ × JX♂	81.00 ± 2.48[b]	0
JL♀ × XJ♂	0	0
JX♀ × JX♂	78.44 ± 7.35[b]	70.56 ± 5.63[a]

1

Table 3(on next page)

Genetic diversity parameters of *Quasipaa*

Notes: “JX,” “JC,” and “ZJ” represent Jinhua *Q. spinose*, Lushan *Q. shini*, and hybrid tadpoles, respectively

Population	number of samples	N_A	N_E	H_O	H_E	PIC	F_{IS}
JX	31	8.44	4.48	0.46	0.53	0.50	0.15
JC	24	3.78	2.20	0.53	0.49	0.42	0.28
ZJ	31	5.33	3.14	0.89	0.66	0.59	-0.36

1

Figure 1

Growth and development of pure and hybrid tadpoles in the three water temperature groups

(A) body length rate

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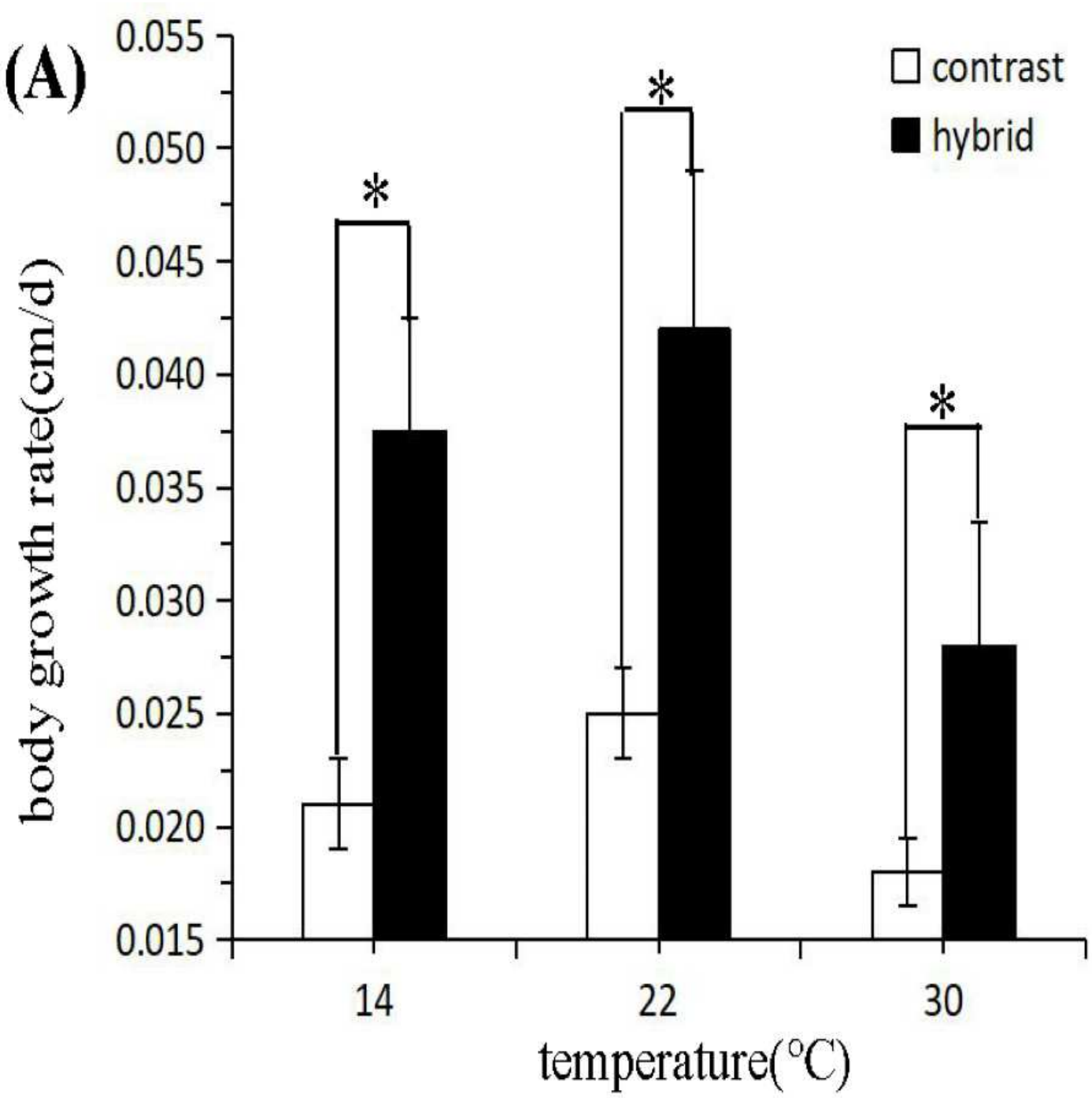

Table 4(on next page)

Analysis of molecular variance (AMOVA) of *Quasipaa* populations

Notes: *means significant difference (P<0.05).

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Source of variation	Sum of squares	Variance components	Percentage of variation
among populations	76.46	0.83	24.08*
within populations	360.73	2.60	75.92*
total variation	437.20	3.43	—

1

Figure 2

Growth and development of pure and hybrid tadpoles in the three water temperature groups

weight growth rate

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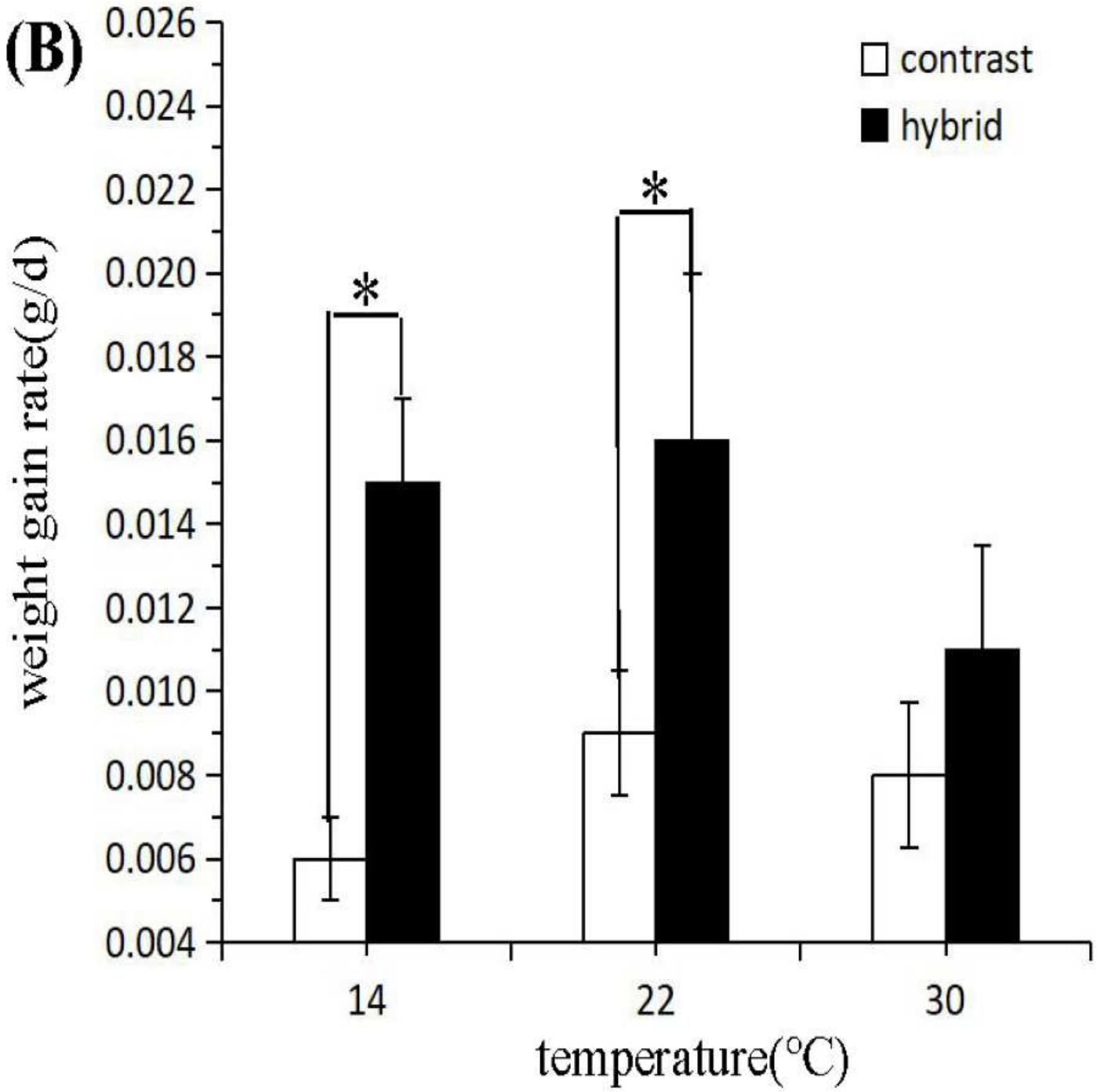

Table 5(on next page)

Bottleneck effect analysis of populations in *Quasipaa*

Notes: **means extreme significant difference (P<0.01).

Population	IAM		TPM		SMM		Mode shift
	one tail	two tail	one tail	two tail	one tail	two tail	
JX	0.632	0.820	0.752	0.570	0.997	0.010**	L-shaped
JC	0.285	0.570	0.410	0.820	0.820	0.426	L-shaped
ZJ	0.001**	0.004**	0.064	0.129	0.410	0.820	L-shaped

1

Figure 3

Swimming speed of hybrid and purebred tadpoles in the three water temperature groups at different time periods

14 °C treatment group

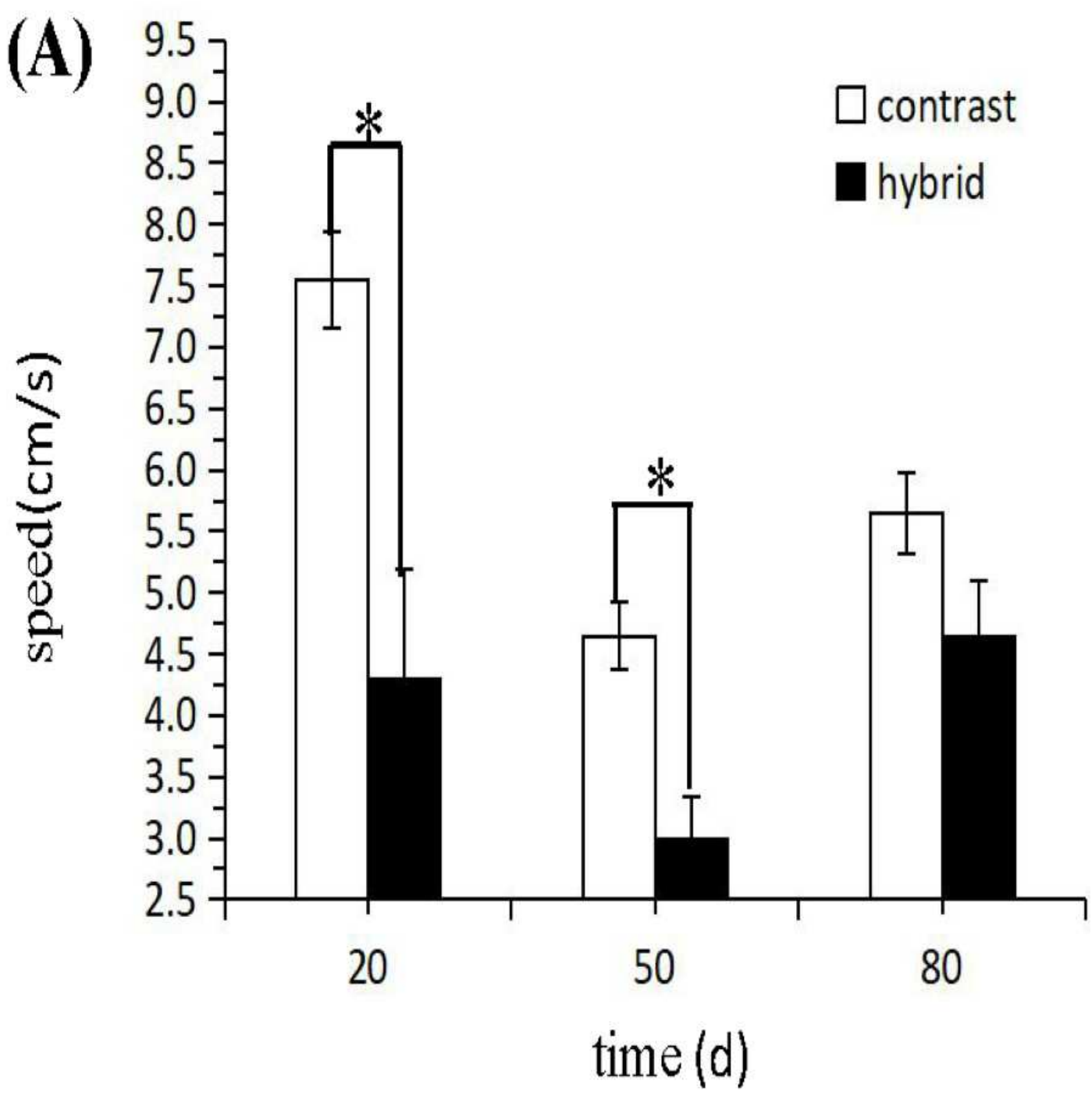

Table 6(on next page)

Genetic differentiation of *Quasipaa* populations based on microsatellite markers

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Population	JX	JC	ZJ
JX	-		
JC	0.14	-	
ZJ	0.32	0.47	-

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Figure 4

Swimming speed of hybrid and purebred tadpoles in the three water temperature groups at different time periods

22 °C treatment group

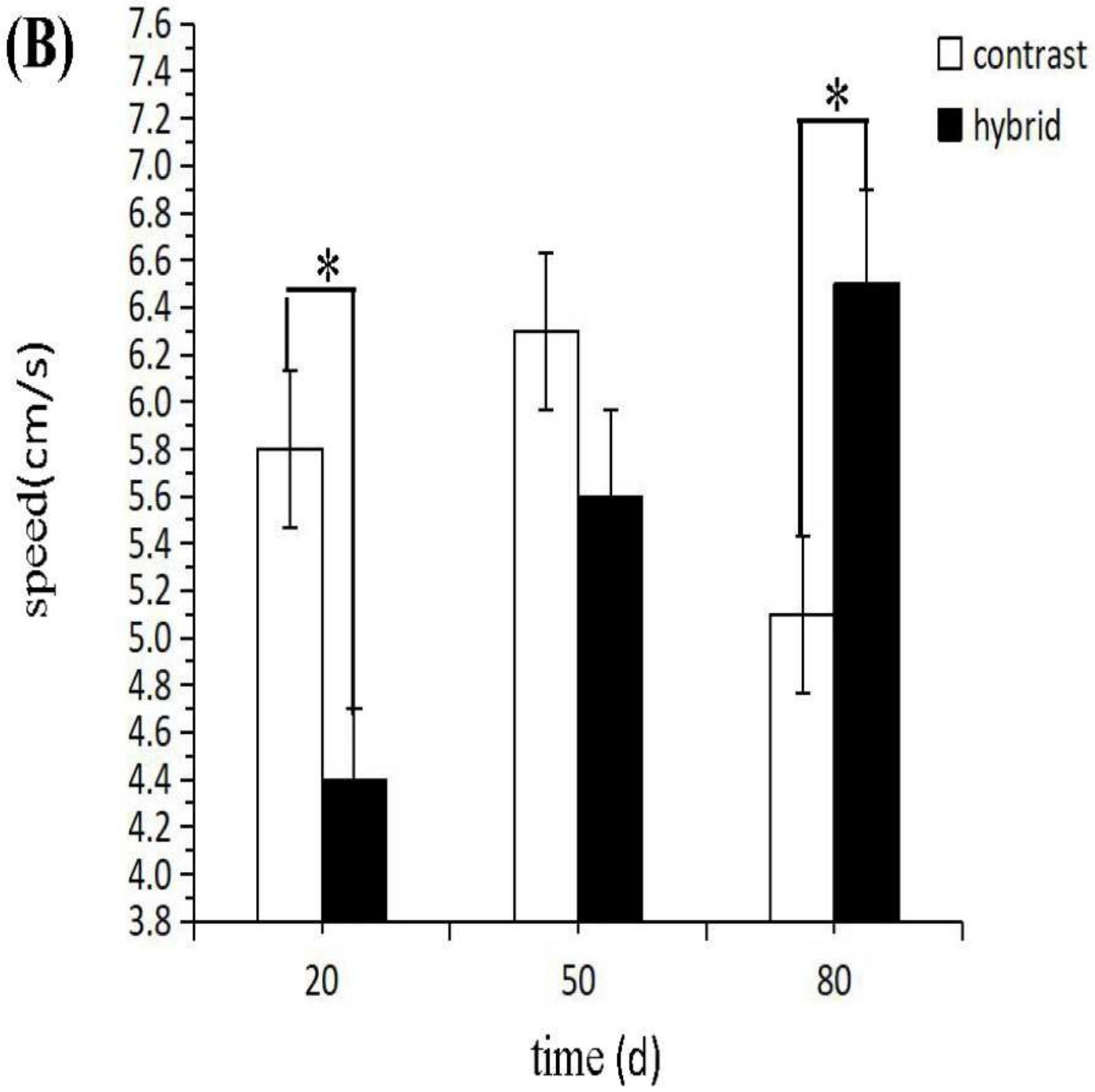

Figure 5

Swimming speed of hybrid and purebred tadpoles in the three water temperature groups at different time periods

30 °C treatment group

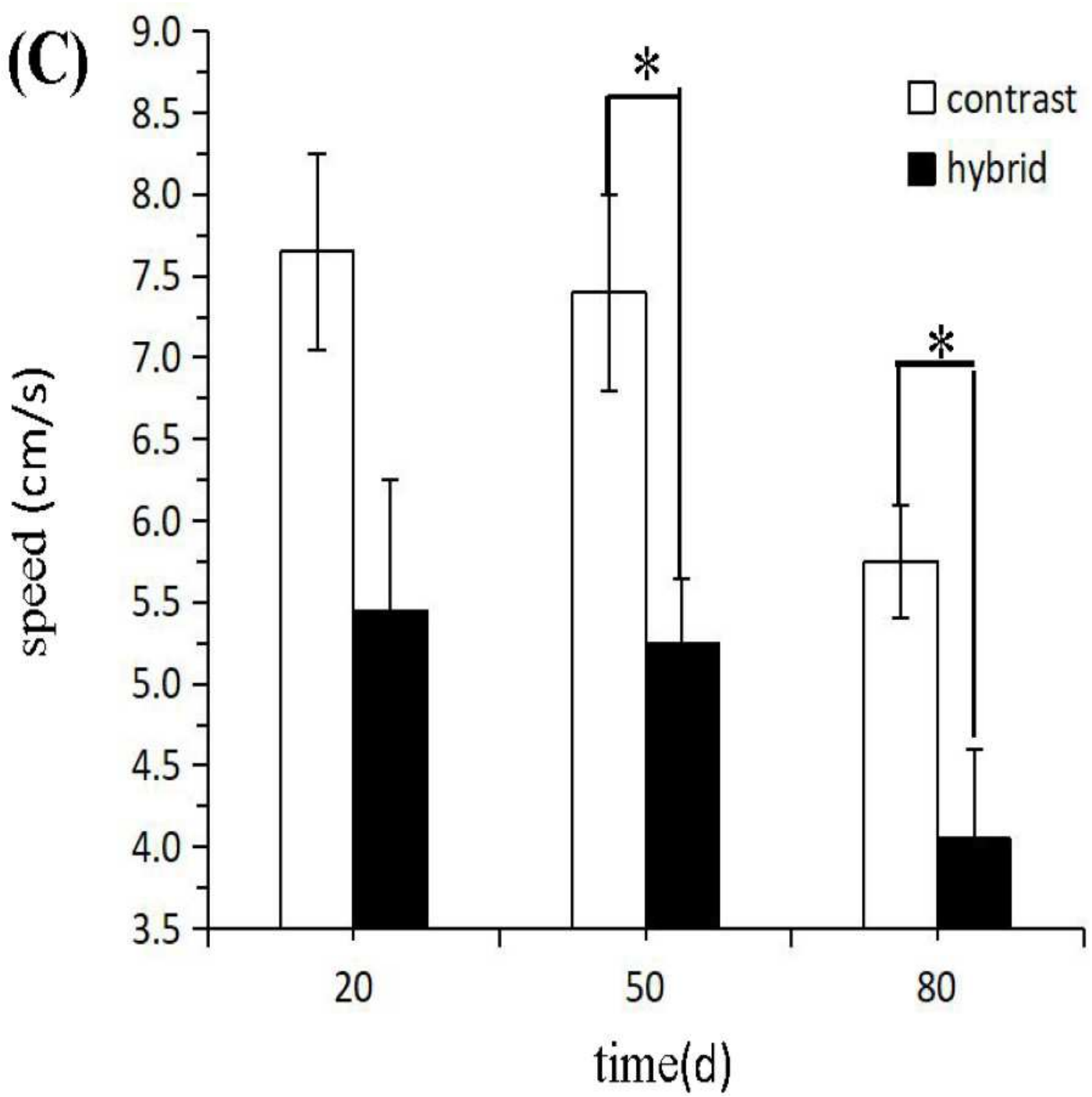

Figure 6

Growth and development of hybrid and purebred tadpoles in the three density groups

body length rate

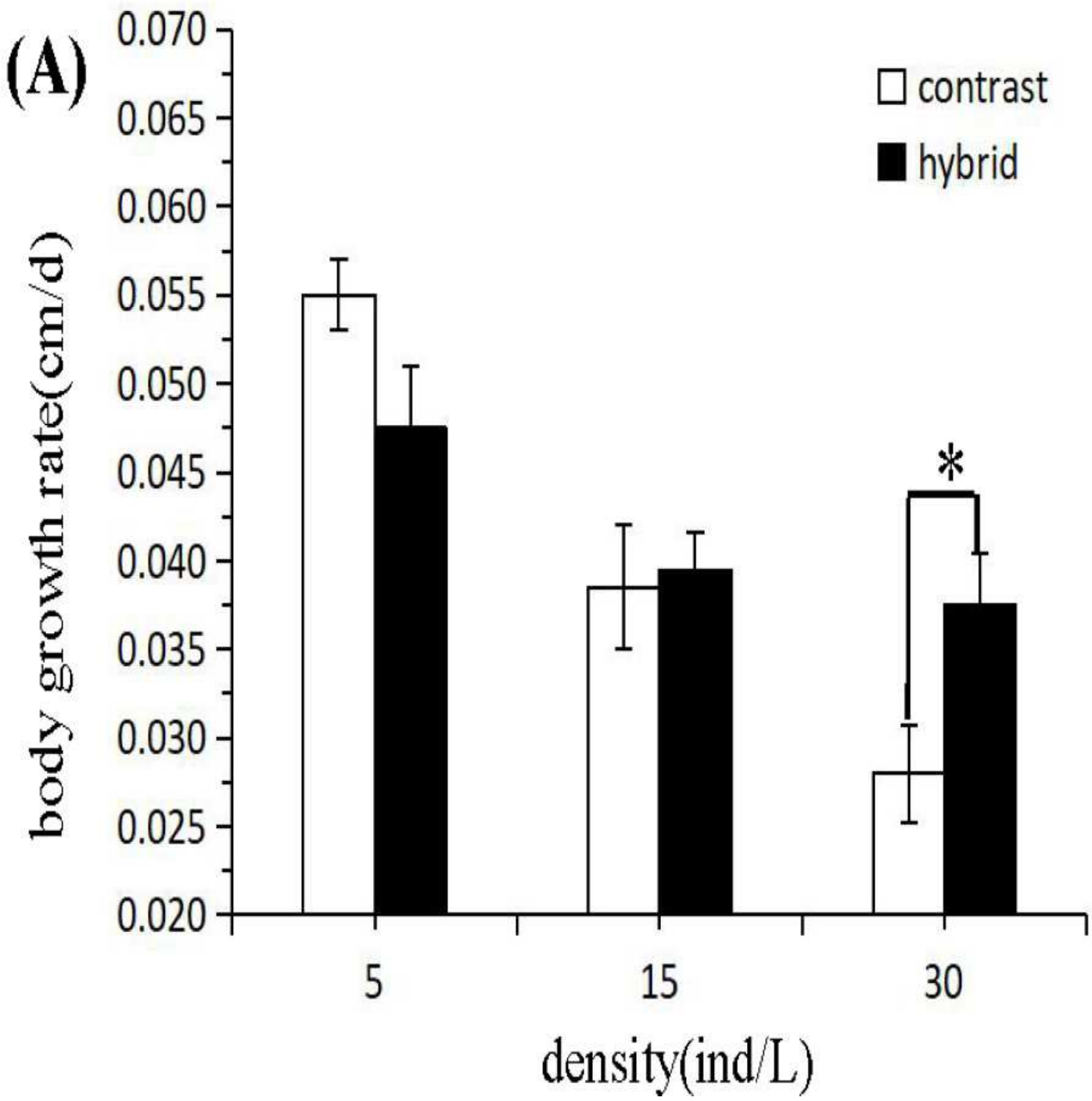

Figure 7

Growth and development of hybrid and purebred tadpoles in the three density groups

weight growth rate

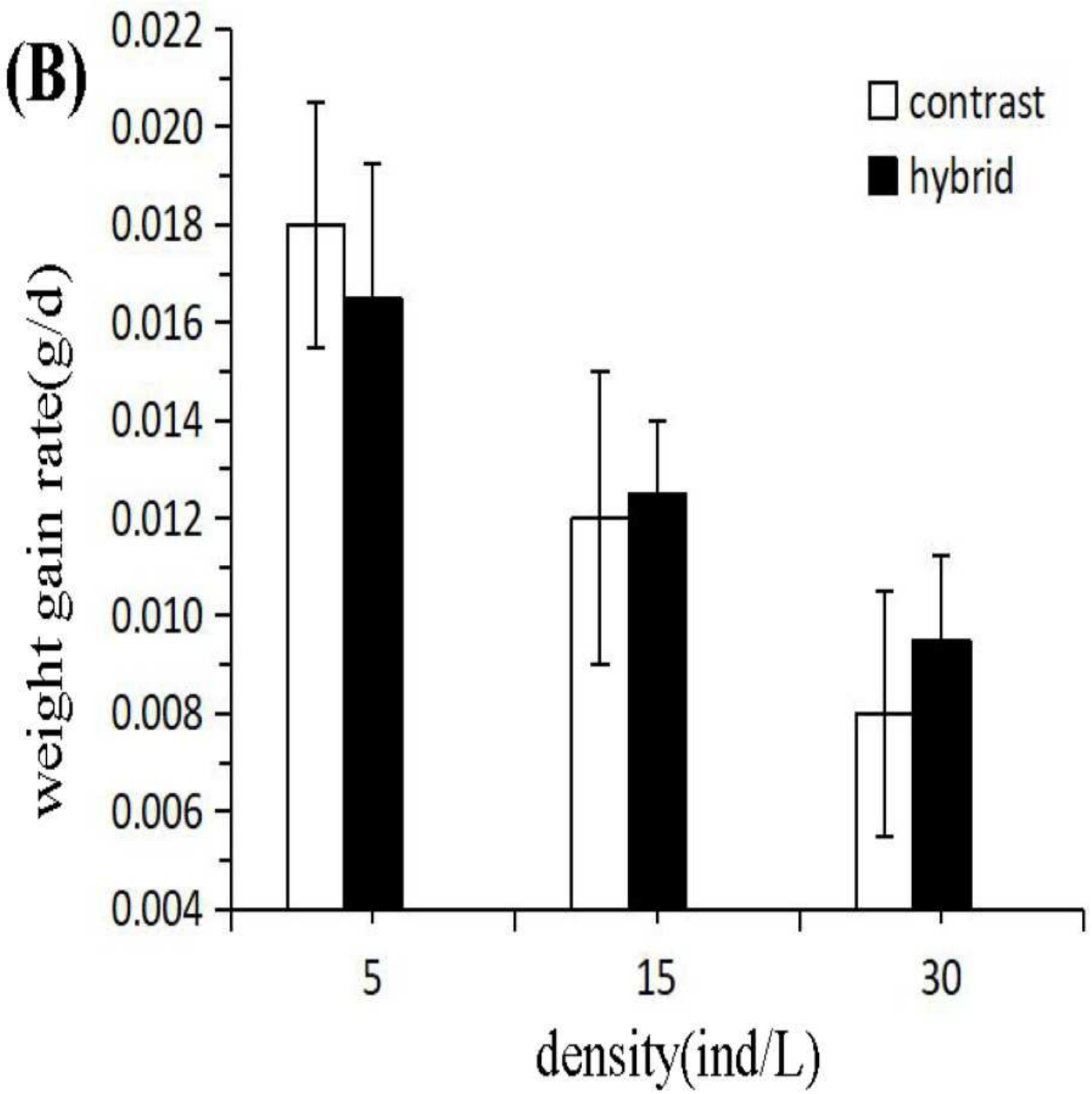

Figure 8

Swimming speed of hybrid and pure tadpoles in the three density groups at different time periods

5 ind/L treatment group

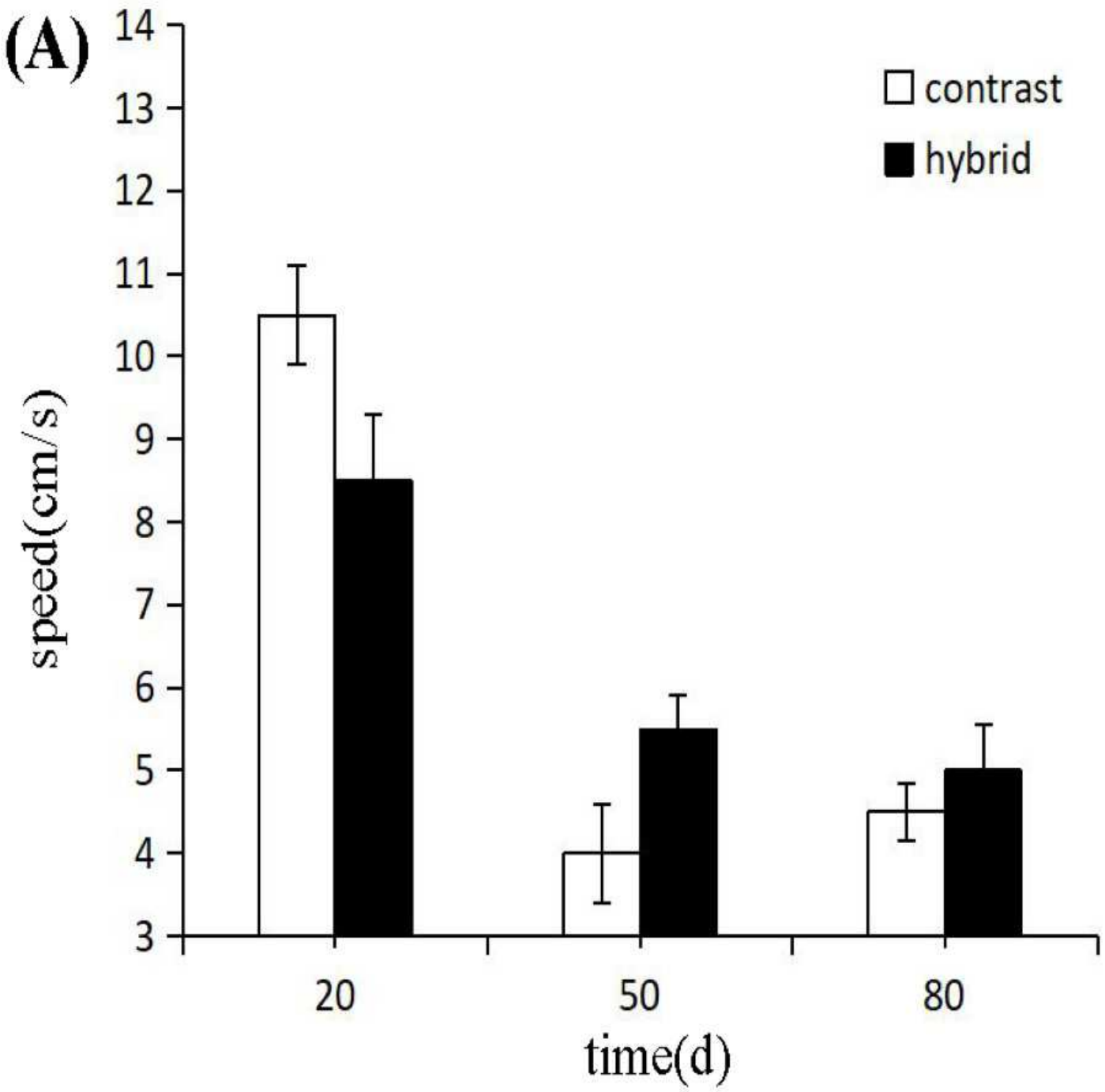

Figure 9

Swimming speed of hybrid and pure tadpoles in the three density groups at different time periods

15 ind/L treatment group

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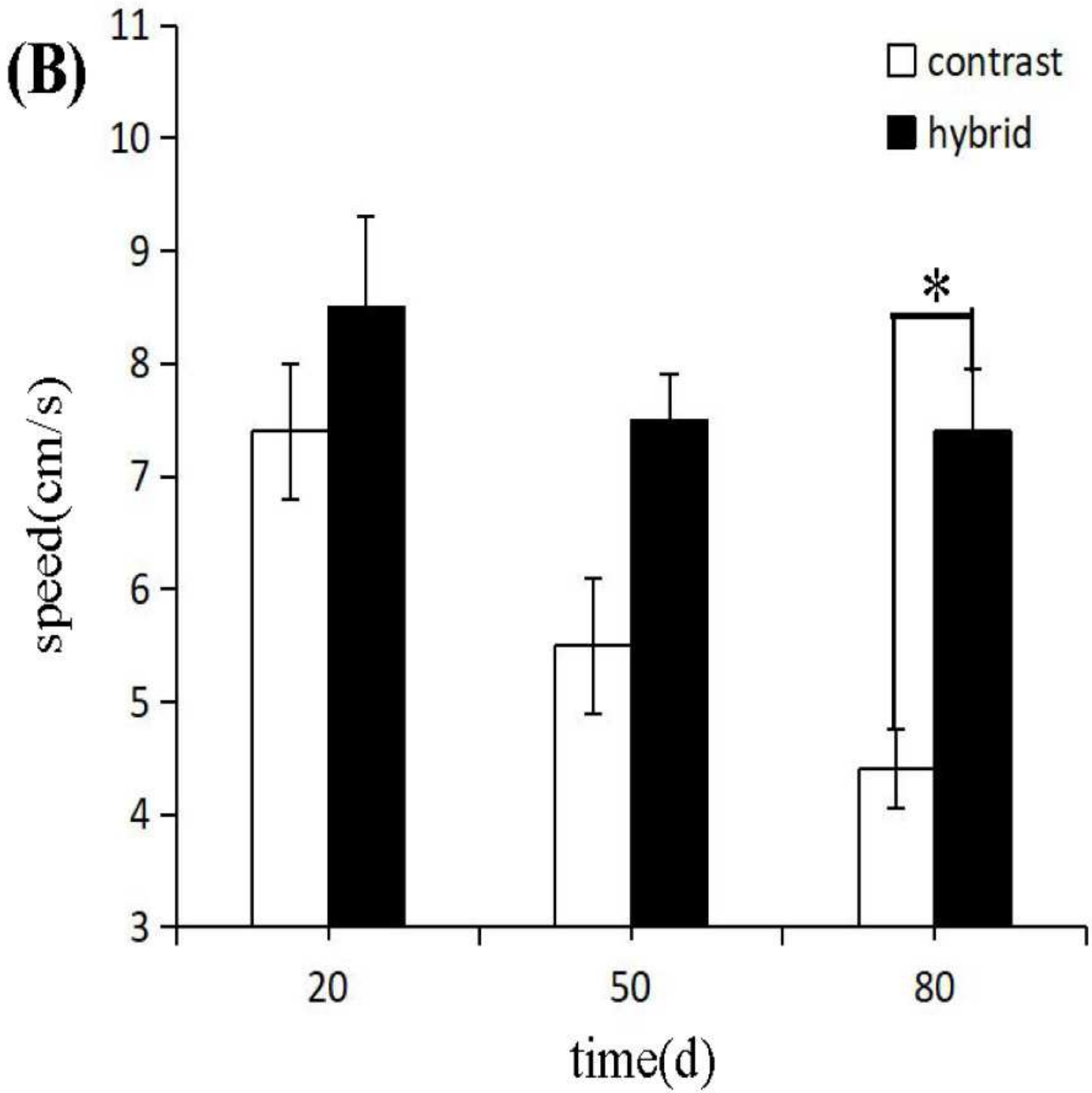

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Figure 10

Swimming speed of hybrid and pure tadpoles in the three density groups at different time periods

30 ind/L treatment group

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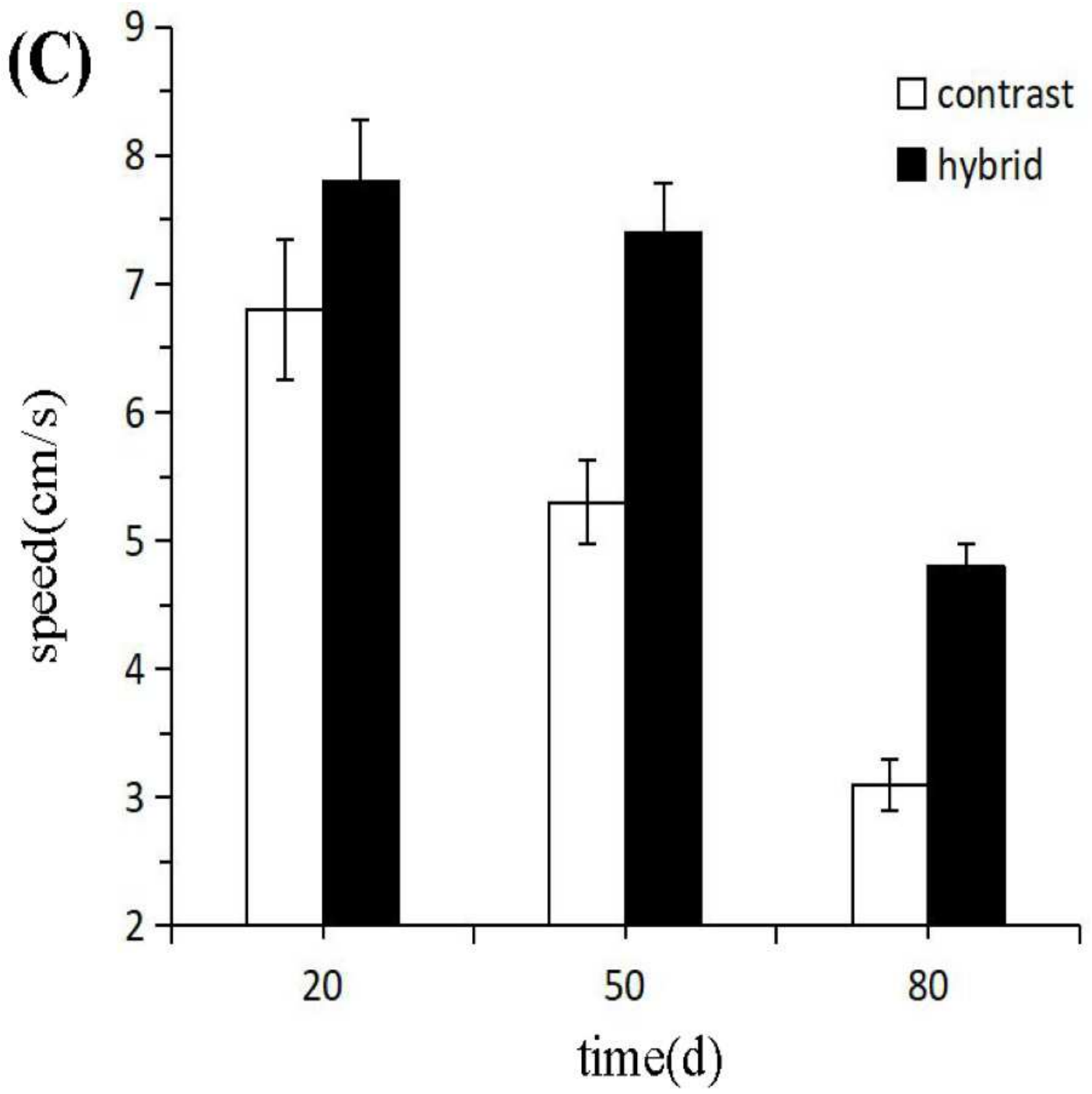

Figure 11

Best number of ancestral cluster (K) according to online tool Structure Harvester

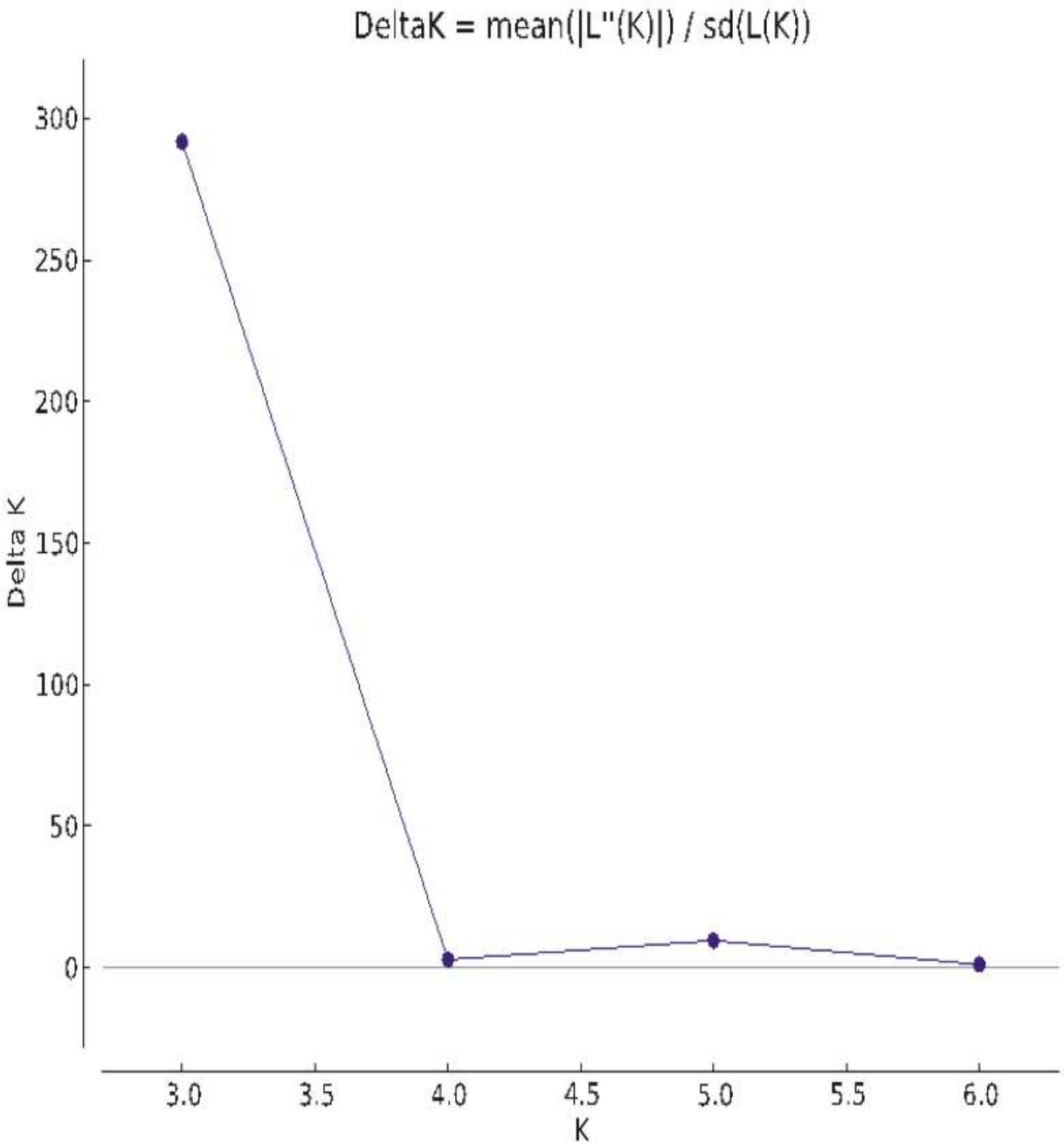

Figure 12

Population structure plots by structure analysis. The sample location for each individual is also indicated

The sample location for each individual is also indicated