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Morphology and Genetic Structure Profile of Farmed Snails *Cornu aspersum aspersum* and *Cornu aspersum maximum* in Greece

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Abstract: The subspecies of the species *Cornu aspersum*, *C. a. aspersum*, and *C. a. maximum* are the dominant farmed species in Greece. The morphological and molecular polymorphism of the two aforementioned subspecies has not been studied in depth. In this study, the polymorphism of snails of the two subspecies derived from seven snail farms throughout Greece was studied using morphological and molecular markers. Firstly, the snail samples of both subspecies were categorized in three shell patterns based on shell color and existence of bands. The conducted population structure analysis revealed three major clusters among the farmed snail populations. As concerns genetic diversity, six loci (Ha5, Ha6, Ha8, Ha9, Ha10, and Ha11) were tested for their polymorphism. Genetic variation was reported within populations rather than among populations. Finally, the obtained data highlighted a common gene pool broodstock for snail farms throughout Greece.

Keywords: farmed snails; *C. aspersum*; shell; polymorphism; heterozygosity; genetic structure

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1. Introduction

Land snails have been considered as ideal organisms for phylogeographical and ecological genetics studies because of their limited dispersal capacity and their habitat requirements [1–3]. Phylogeographic studies have focused on historical factors affecting species distribution and population through repeated cycles of population elimination and isolation in regression, and led to the creation of biogeographic barriers [4,5]. The species *Cornu aspersum* (*Helix aspersa*) (Muller, 1974) has two infraspecific taxa, *C. a. aspersum* and *C. a. maximum*, which split in the mid-to-late Pliocene [6,7] and live sympatrically in North Africa, while *C. a. aspersum* occurs in America, Australia, New Zealand, and South Africa [8]. Among species widely distributed across the Mediterranean basin, the land snail *C. a. aspersum* has proved to be a suitable model to discover phylogeographical patterns across North Africa and surrounding regions of the Western basin, and to evaluate hypotheses leading to population differentiation [9]. Studies based on molecular markers have described two divergent lineages for *C. a. aspersum*, named "East" and "West" according to their geographical location in North Africa [6,10,11], and since the Holocene, the species *C. aspersum* has successfully colonized a large range of man-disturbed habitats in Western Europe considered as an important pest in lands. Although the exact geographical origin of the species remains unresolved, the first phylogeographical scenario, based on mitochondrial DNA variation of European and North African populations, assumes that ancestral populations of the species *C. a. aspersum* would have dispersed in the western Mediterranean through microplate tectonics from Oligocene [12]. Many phylogenetic studies [5,9,13,14] have been carried out for the subspecies of *C. a. aspersum* while the origin of the commercial species *C. a. maximum* is unknown apart from few historical references [11]. The two subspecies live sympatrically in North Africa, while *C. a. aspersum* occurs in America, Australia, New Zealand, and South Africa [8].

The comparison of anatomical features of organisms has been a central element in biology for centuries [15] and can be used for studying polymorphism. Polymorphism is a basic characteristic of shell morphology, which preserves the onto-genetic record of growth, and is now the main subject of a sub-field of morphometrics [16]. The shell of *C. a. aspersum* is characterized by pronounced polymorphism [17], a phenomenon which can be found in several land snail species including *Cepaea hortensis*, *Theba pisana*, and *C. a. aspersum* [18,19]. The limited dispersal capacity of terrestrial snails [1] explains the high genetic diversity and the polymorphism of the shell [20]. The coloration of the shell base varies from pale yellow to dark brown, and dark elongate bands of various types and colors are present in the shell. Differences in size, weight, and other morphometrical characteristics of the shell can be explained by the species, the age of snails, the period of collection, the breeding conditions and diet as concerns the farmed subspecies *C. a. aspersum* and *C. a. maximum* as well as wild species [21–24]. More specifically, several studies attribute this polymorphism to predation or climatic effects [25]. Dark banding occurs mainly in snails growing at low temperatures, while in snails growing at temperatures above 25 °C the color of the banding becomes red [26]. The assumption of a higher warming capacity in darker snail shells should be regarded with caution, and alternative possibly selecting factors and correlations have been proposed, among which are the humidity and the higher occurrence of parasites under humid conditions in northern and/or sheltered habitats.

In addition, geographic polymorphism in edible snails of *C. a. aspersum* has been studied by isozyme-allozyme analysis, mitochondrial DNA analysis and the recording of shell morphological characteristics in snail populations from Western Europe and North Africa [10,27,28]. In case of *C. a. aspersum*, polymorphism has been studied based on morphological, biochemical, and molecular markers. In Greece, the polymorphism of *C. a. aspersum* has been studied by isozyme–allozyme analysis [29]. Snails of this subspecies from 24 sites in Greece and Cyprus were classified based on morphological markers into three populations [29]. In addition, isozyme–allozyme and mitochondrial DNA studies of snails from different regions of South Africa were carried out and they were distinguished into two populations, namely, east and west [6,10,28]. Polymorphic markers such as microsatellites (SSRs) replaced allozymes or mitochondrial DNA in population and hybridization studies. Additionally, microsatellites are the most commonly used molecular receptors in population genetics due to their repeatability. Many studies about fish population structure have been conducted using the aforementioned DNA markers [30], though the population structure in the marine environment is sometimes cryptic due to complex demographic expansion and distribution of the species [31].

Maintaining genetic diversity in captive populations is initiated with a small number of individuals from wild remnant populations [32]. Genetic heterozygosity in wild, unmanaged animal populations is often associated with protection against infectious disease [33]. In species such as sheep, studies have been focused on investigations into the heritability or genetic susceptibility to footrot in sheep [33,34]. Additionally, the genetic basis of the positive correlations observed between multi-locus heterozygosity at allozyme loci and fitness-related traits such as growth rate, viability, feeding rate, or fecundity is reported in several organisms [35,36]. As concerns snails, there is a lack of data mainly for land snails. Multiple-locus heterozygosity at four polymorphic loci under low-density and high-density laboratory conditions was examined with protein electrophoresis in the land snail *Otala lactea* [37]. Snails with more heterozygous loci did not grow faster under high- or low-density conditions, while significant differences in the initial weight among genotypes occurred at the locus 6-phosphogluconic dehydrogenase, but no differences in weight gain among genotypes occurred in the laboratory growth experiments. No differences were found in weight gain or multiple-locus heterozygosity between banded and unbanded snails. Correlations between heterozygosity and several fitness measures including juvenile survival, survival to sexual maturity, and fecundity, and considered trends in the inbreeding coefficient (F_{IS}) over generations were examined for *Achatinella lila* snails after their population decline. Snails with higher measures of heterozygosity had more offspring,

and third-generation offspring with higher measures of heterozygosity were more likely to reach maturity.

While snail species can be identified morphologically when fresh, when they are processed other techniques must be used, which prevent consumer fraud. Food authenticity is affected by genetic background and geographical origin [38]. The species, the age, and the procedure followed during breeding might affect the unique qualitative characteristics of snails and their authenticity. More specifically, in the species *Helix pomatia*, *Helix lucorum*, and *Achatina fulica*, using four restriction enzymes and by testing 12S rRNA and 16S rRNA fragments, researchers were able to distinguish the three snail species [39]. The body morphology and the free amino acid (FAA) profile variability were used for stock identification among farmed rainbow trout Oncorhynchus mykiss populations [40].

Snail food products are very famous and consumed throughout the world because of their high nutritional value and their unique qualitative characteristics [24]. Our goal was to investigate the morphological polymorphism and describe, for the first time, the genetic structure of farmed snails, which are a food product consumed worldwide [21]. The results obtained in this research are a part of a research project focused on snail farming in Greece. Our goal was to investigate the snail farms and the factors affecting snail quality. *C. a. aspersum* and *C. a. maximum* snails, used for polymorphism studies, are the main farmed subspecies in Greece [21]. Here, we chose to use snail populations of two farmed subspecies *C. a aspersum* and *C. a. maximum* in order to analyze their genetic structure and investigate their authenticity.

2. Materials and Methods

2.1. Snail Collection and Tissue Isolation

According to the sampling procedure, a total of 160 snail specimens of the two infraspecific taxa of the species *C.aspersum* were used [7]. Forty *C. a. aspersum* one-hundred and twenty 120 *C. a. maximum* snails were collected from 7 snail farms in Greece (Figure 1).

The 160 snails from the 7 snail farms comprised 8 populations with 20 snails each. From the farms S1, S2, S5, S6, and S7, we collected snails of *C. a. maximum* and the populations were coded as SDN, SRT, VOL, AGR, and VSL, respectively. Snails of the species *C. a. aspersum* were collected from farm S4 and the population was coded as OMR. In farm S3, both species were bred and the populations of *C. a. aspersum* and *C. a. maximum* were coded as KNT1 and KNT2, respectively. All the information concerning age, farm, and sample generators and productive procedure such as farm type, diet, and climate were collected. The data about generators of snail farms, sample generators, and production procedures are shown in Figure A1.

The polymorphism was assessed for farmed snails reached the appropriate marketable size and their age ranged from 126 to 210 days. The sample generators mainly derived the previous production period of the snail farm based on the operation years of the farm. Despite snail farm S4, all the other farms had snails of marketable size from another snail farm as first generators. As concerns farm type, only two snail farms belonged to the list of open-field farms while farms with the subspecies *C. a. maximum* reported higher biotic load (Figure 2). Additionally, all the farms of the sampling procedure used mainly a compound diet for snail and less the combination of compound diet with plants, were equipped with a low-pressure cooling system. All snails *C. a. aspersum and C. a. maximum* were transported to the Laboratory of Ichthyology-Hydrobiology 2 days after their collection, in December 2017.

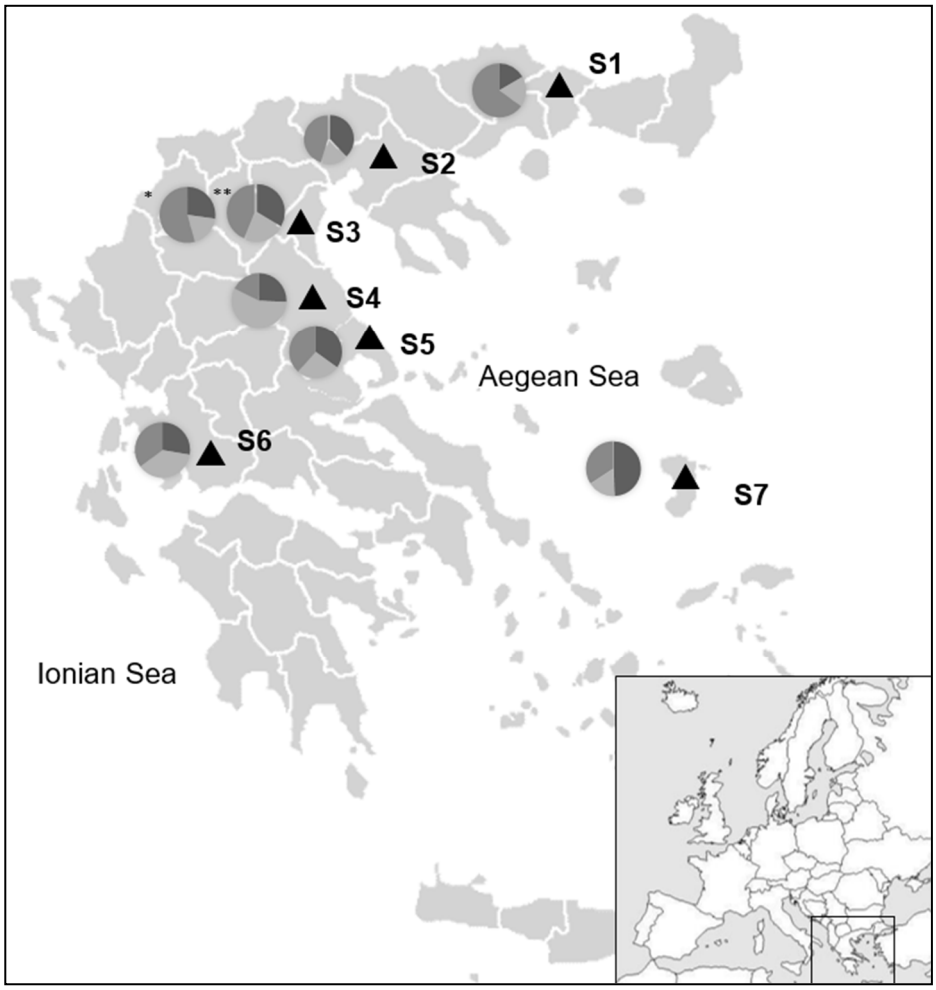

Figure 1. Map of Greece showing the sampling snail farms of *C. a. aspersum* and *C. a. maximum*. S1: Sidini (Xanthi) 41°04′35.8″ N 25°01′22.6″ E, S2: Souroti (Thessaloniki) 40°28′01.7″ N 23°05′27.1″ E, S3: Kondariotissa (Pieria 40°13′21.0″ N 22°27′16.9″ E, S4: Omorphochori (Larissa) 39°39′53.2″ N 22°29′10.5″ E, S5: Alli Meria (Volos) 39°22′13.2″ N 22°58′40.1″ E, S6: Agrinio 38°37′36.3″ N 21°25′23.2″ E and S7: Vasileoniko (Chios) 38°19′44.8″ N 26°06′47.7″ E. Pies denote the presence of three putative clusters (K = 3) (white, grey color, black color) in each population revealed by STRUCTURE analysis.* population of *C. a. aspersum*, ** population of *C. a. maximum*.

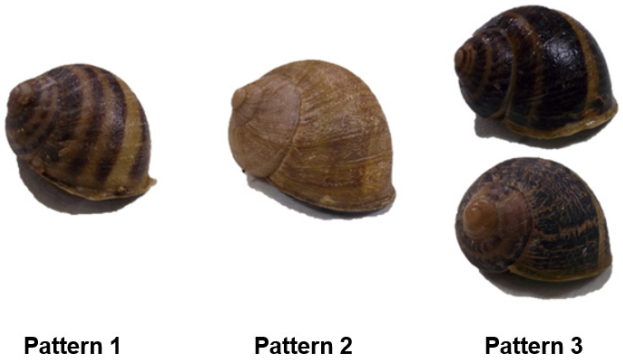

Figure 2. The three shell patterns: p1 = light color shell with bands, p2 = light color shell without bands and p3 = black color shell.

The collected snails were categorized according to their shell color and the existence of bands in three shell patterns: p1 = light color shell with bands, p2 = light color shell

without bands and p3 = black color shell (Figure 2). Finally, the mantle color was assessed as dark, light or intermediate. The morphological characteristics such as shell diameter (D), shell height (H), aperture diameter (d) and animal weight (W) were assessed for all snails [21,41]. All anatomical analyses were carried out immediately. After the removal of shell, the weight of the foot-head mass (W_f), referred as fillet [25], the weight of visceral mass (W_v) and shell (W_s) were also assessed. Part of fillet was stored at –20 °C for later molecular analysis.

2.2. DNA Extraction and PCR Amplification

Total cellular DNA was isolated from muscle samples of *C. a. aspersum* and *C. a. maximum* snails following a modified phenol-chloroform protocol after [42].

A panel of 6 microsatellite DNA loci, Ha5, Ha6, Ha8, Ha9, Ha10, and Ha11 [10] was tested (Table 1). A multiplex PCR Kit with hot start Taq (KAPA2G Multiplex Mastermix, KAPA BIOSYSTEMS) was used for the DNA amplifications. The primers were divided into three multiplex groups according to fluorescent primer pigment.

Table 1. Sequence of primers: Ha5, Ha6, Ha8, Ha9, Ha10, and Ha11 [10].

Primer	Sequence			Pigment
Ha5	F:GTGTGACACACTGCCCTGGA R:CAATGGCAAACTACTGAAAGCAA	$(TG)_{19}$	117–207	FAM
Ha6	F:TTATCCGCTTGATATATCCT R:ACTCGTACATGGTTGAAAAC	$(GA)_{23}(GGA)_4$	145–215	HEX
Ha8	F:AGTTTGCTGGTTTGTACACTCG R:CGTTTTTAGCTCTTGAATACGG	$(CA)_{14}CGTG(CA)_3AGATG(CA)_2$	152–210	FAM
Ha9	F:AGCTAACCCACACTCAGATTT R:AGCCAGCTAATATGTTTGGA	$(TG)_5 \ldots (CA)_{20} \ldots (AT)_6$	108–172	TAMRA
Ha10	F:GCGTTCAATGTAGTTTATGTGCG R:GAGAACATGCATACAAACAAACATG	$(CA)_6(CGCA)_3(CA)_4TACACG(CA)_{14}$	211–253	TAMRA
Ha11	F:CGTGTACTACTGGGCAACGT R:ACGGAAAGAGACAGAAAGTGAG	$(TC)_2ACTGTTCC(TC)_{33}$	175–240	HEX

The PCR cycling profile was 95 °C for 3 min; 30 cycles of 94 °C for 1 min, annealing temperature for 50 s and 72 °C for 50 s; and 72 °C for 8 min. The length of PCR products was verified by electrophoresis on 1.5% agarose gel containing 0.5 μg mL^{-1} ethidium bromide. Amplified DNA products were screened on an ABI 3500 DNA Analyser (Applied Biosystems). Each specimen's alleles were scored by the software STRAND 2.0 [43].

2.3. Data Analysis

Morphometric data were analyzed statistically using the analysis program SPSS [44]. To assess the significance of differences of morphometrical characteristics among groups, One-Way-ANOVA test and independent-samples *t*-test were applied. Post-hoc comparisons were performed using the Tukey test ($p < 0.05$). Principal component analysis (PCA) was applied for the morphological data of all snails used in this study. Exact tests for Hardy–Weinberg equilibrium and the linkage disequilibrium method with 10,000 dememorization steps followed by 1000 batches with 10,000 iterations per batch were carried out using GENEPOP 3.4 [45]. Expected heterozygosity (H_{EXP}) and observed heterozygosity (H_{OBS}) were estimated using GENEPOP 3.4 [45]. Fixation indexes F_{ST} were measured by genetic differentiation and F_{IS} using the formulations described by Weir and Cockerham [46]. GenAlex 6.5 software [47] was also used for the estimation of statistical significance of pairwise differentiation, taking into account the composite haplotype frequencies [47].

Analysis of molecular variance (AMOVA) was applied using the GenAlex 6.5 [47]. Population structure was further assessed using STRUCTURE 2.3 [48] assuming correlated allele frequencies. Three independent repeats were run for each value of K ($1 \leq K \leq 9$). Following test runs, the burn-in length and length of simulation were set at 1,000,000 and 3,000,000 repetitions, respectively. STRUCTURE HARVESTER [49] was used to assess the likelihood value of the different K values and to implement the ΔK method [50] reflecting the highest hierarchical level of structuring [51]. Using software IR-macro, internal relatedness IR Homozygosity by loci (HL) and H_{OBS} were estimated and their correlations with morphometrical characteristics (D, H, d) were assessed by estimating Pearson's coefficient.

3. Results

The morphological characteristics of the shells (D, H, d) and the weights of whole snails (W), fillets (Wf), visceral mass (Wv), and shells (Ws) were assessed for the 20 snails of each population and mean values of these parameters and differences among populations are presented in Table 2.

Table 2. Morphological characteristics of the population of the two snail species, *C. a. aspersum* and *C. a. maximum*. Results are expressed as mean values ± S.D. (n = 20). For *C. a. aspersum* data, *t*-test was performed and for *C. a. maximum*, one-way ANOVA followed by Tukey test and Kruskal–Wallis test were performed. Data within the same column for each species that do not share a letter are significantly different ($p < 0.05$). D = snail shell diameter, H = snail shell height, d = snail shell aperture diameter, W = weight of whole raw snail, W_f = weight of raw fillet, W_v = weight of visceral mass, and W_s = weight of shell. Small letters denote statistically significant differences ($p < 0.05$).

Subspecies	Population	D (mm)	H(mm)	d(mm)	W(g)	Wf (g)	Wv(g)	Ws(g)
C. a. aspersum	KNT1	29.36 ± 1.58 ^{a}	28.74 ± 1.04 ^{a}	14.50 ± 1.80 ^{a}	6.52 ± 0.83 ^{a}	0.80 ± 0.12 ^{a}	3.26 ± 0.49 ^{a}	2.46 ± 0.52 ^{a}
	OMR	27.20 ± 1.84 ^{b}	26.23 ± 1.33 ^{b}	13.61 ± 0.92 ^{a}	5.22 ± 0.78 ^{b}	0.72 ± 0.08 ^{b}	2.84 ± 0.60 ^{b}	1.67 ± 0.41 ^{b}
	Statistics	P = 0.00, T = 3.98	P = 0.00, T = 6.66	P = 0.058, T = 1.98	P = 0.00, T = 5.06	P = 0.022, T = 2.40	P = 0.00, T = 5.28	P = 0.019, T = 2.45
C. a. maximum	SDN	38.08 ± 2.08 ^{b}	36.83 ± 1.40 ^{b,c}	19.98 ± 1.57 ^{b}	12.36 ± 1.92	2.31 ± 0.26	3.99 ± 0.92	6.06 ± 1.20 ^{c}
	SRT	35.37 ± 2.14 ^{c}	34.50 ± 1.96 ^{d}	18.17 ± 1.59 ^{c}	10.64 ± 1.69	2.10 ± 0.19	3.62 ± 0.90	4.92 ± 1.06 ^{c}
	KNT2	37.74 ± 2.08 ^{b}	36.26 ± 1.96 ^{c}	20.19 ± 2.74 ^{b}	12.26 ± 2.03	1.77 ± 0.36	5.33 ± 0.90	5.16 ± 1.30 ^{b}
	VOL	39.97 ± 2.57 ^{a}	38.26 ± 1.98 $^{a.b}$	21.42 ± 1.58 $^{a.b}$	14.06 ± 2.09	2.29 ± 0.28	5.68 ± 1.22	6.09 ± 1.43 ^{b}
	AGR	41.78 ± 1.59 ^{a}	38.86 ± 1.81 ^{a}	22.24 ± 1.49 ^{a}	17.90 ± 2.02	3.00 ± 0.41	7.71 ± 1.57	7.19 ± 1.30 ^{a}
	VSL	37.83 ± 1.55 ^{b}	35.81 ± 1.63 $^{c.d.}$	18.28 ± 1.37 ^{c}	12.13 ± 1.85	1.94 ± 0.13	6.08 ± 1.43	4.10 ± 0.68 ^{b}
	Statistics	P = 0.00, F = 23.17	P = 0.00, F = 15.98	P = 0.00, F = 16.76	P = 0.00, H = 61.85	P = 0.00, H = 73.73	P = 0.00, H = 51.68	P = 0.00, F = 31.34

According to Table 2, KNT1 and OMR snails presented significant differences as concerns the size and mass. The diameter of the aperture was the same in two populations of *C. a. aspersum*. *C. a maximum* snails presented significant differences as concerns the morphometrical characteristics of shells and mass of shells according to one-way ANOVA ($P < 0.05$). According to the PCA results (Figure A2), based on the morphometrical characteristics of the shell and mass of whole snail, fillet, visceral mass, and shell of the farmed snails among populations, the amount of variation explained by each axis was 86.7% for the first component and 7.4% for the second component. According to the PCA results for each subsecies (Figure 3), the amount of variation explained by each axis is 11.45% for Coordinate 1 and 8.29% for Coordinate 2 for *C. a. aspersum* and 6.79% for Coordinate 1 and 4.8% for Coordinate 2 for *C. a. maximum* (b).

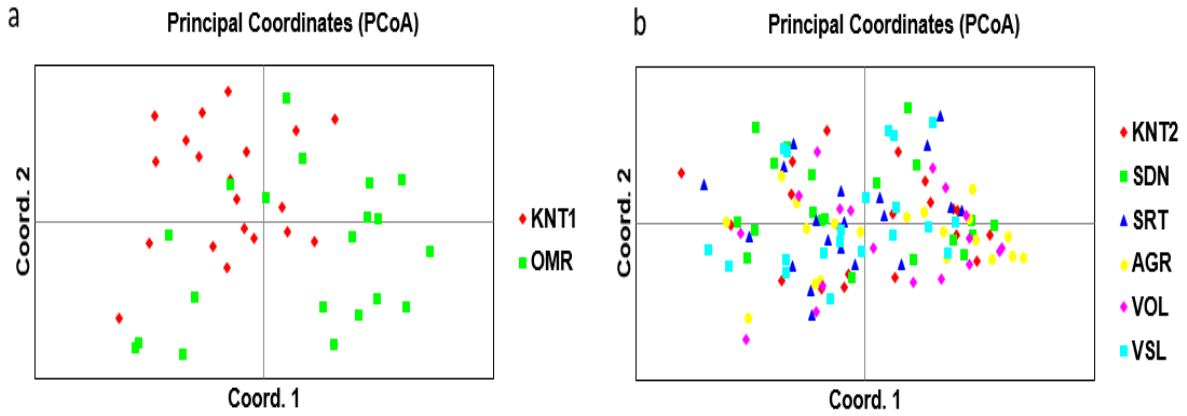

Figure 3. Principal coordinate analysis (PCoA) of the farmed populations. The amount of variation explained by each axis is 11.45% for Coordinate 1 and 8.29% for Coordinate 2 for *C. a. aspersum* (**a**) and 6.79 % for Coordinate 1 and 4.8% for Coordinate 2 for *C. a. maximum* (**b**).

As concerns shell color and bands, snails from both subspecies were categorized in three patterns; p1 was for light = color shells with bands, p2 for light-color shells without bands, and p3 for black-color shells. No significant differences (P = 0.807, F = 0.0535) were reported among and within populations of the two farmed subspecies of *C.aspersum* as concerns the shell patterns. According to results, the majority of both populations of *C. a. aspersum* had light-colored shells with bands. In the population KNT1, 10% of the snails had dark shells, while 15% had light shells without bands. In contrast, the OMR snails had no light-colored shells without banding, as 90% of the snails belonged to p1 and only 10% belonged to p3. In all *C. a. maximum* populations, the majority of snails (80–95%) were categorized as p1 because they had light-colored shells with bands. According to the same figure, the snails of KNT2 were the only ones that did not have a light-colored shell without bands. In the other five populations, p2 was represented by 5–20% of the snails. Finally, the SRT and KNT2 populations were the only ones in which 10% of the snails presented with p3 shells.

The mantle colors were categorized as light, intermediate, and dark. According to the results, the highest percentage (65%) of *C. a. aspersum* snails of the population of KNT1 and OMR snails had light-colored mantles. Only 35% of the KNT1 snails belonged to a farm that also rears *C. a. maximum* snails, which showed an intermediate mantle colors. In five of the six populations, the snails as a whole had black mantles. A small percentage (15%) of the VSL snails coming from a farm that formerly also reared *C. a. aspersum* had white mantles.

Regarding the results of Table 3, pairwise F_{ST} for *C. a. aspersum* was significant (P = 0.01). All pairwise F_{ST} for *C. a. maximum* populations were not significant. The use of software STRUCTURE 2.3 unraveled three putative clusters (K = 3) among the eight populations considering the membership coefficients which sum to 1 for each individual. The vast majority of populations appeared to have individuals in all clusters.

According to the ANOVA results obtained, 100% of the overall genetic diversity was attributed to be within populations in both subspecies (Table 4). The genetic variation at each locus for each population of both farmed subspecies, *C. a. aspersum* and *C. a. maximum*, is reported in Table 5 (including H_{OBS}, H_{EXP}, number of alleles, and F_{IS}).

Table 3. F_{ST} pairwise values of the farmed snail populations of the two subspecies. An asterisk indicates significance with alpha set to 0.05 after Bonferroni correction.

C. a. aspersum		KNT1				
	OMR	0.034 *				
		KNT2	SDN	SRT	AGR	VOL
	SDN	−0.0065	0			
	SRT	−0.0026	−0.0013	0		
	AGR	0	0.0095	0.0082	0	
	VOL	−0.0014	0.0053	0	−0.0027	0
	VSL	−0.0016	0.0009	−0.0021	0.0088	0.0092

Table 4. Analysis of molecular variance (AMOVA) among and within populations of the subspecies *C. a. aspersum* and *C. a. maximum*. The F_{ST} index represents the sum of variation among populations and variation within populations divided by the total variation.

			C. a. aspersum		
	df	Sum of squares	Percentage of variation	Fixation index	
Among populations	1	0.175	0	F_{ST} = 0.030	P = 0.001
Within populations	38	10.7	100		
Total	39	10.875			
			C. a. maximum		
	df	Sum of squares	Percentage of variation	Fixation index	
Among populations	5	1.517	0	F_{ST} = 0.002	P = 0.197
Within populations	114	51.450	100		
Total	119	52.967			

In Table 5, as concerns *C. a. aspersum*, the farmed population OMR snails showed the highest H_{OBS} values for all loci while F_{IS} values for all loci of KNT1 snails were higher than those of OMR snails. Locus Ha6 presented the highest number of alleles (23) and the highest H_{OBS} (0.650). As illustrated in Table 5, the farmed populations of *C. a. maximum* showed H_{EXP} values ranging from 0.88 to 0.91 and H_{OBS} values ranging from 0.57 to 0.65. For loci Ha6 and Ha11, *C. a. maximum* had the highest number of alleles (26). *C. a. maximum* snails from Kondariotissa (KNT2), where both subspecies were bred, had the lowest value of F_{IS} (0.483) among all loci.

According to Figure 4, the IR value (0.42) of *C. a. aspersum* belonging to P2 was the highest, while no significant differences were reported (P = 0.6, F = 5.12). Although higher IR (0.36) was observed in P1 snails, no significant differences were reported (P = 0.3, F = 1.20) for *C. a. maximum* snails. In both subspecies, P3 snails had the lowest values of IR. According to Pearson's values for the subspecies *C. a. aspersum*, shell diameter (D) had a positive correlation with shell height (H) and aperture diameter (d) (0.589 and 0.648, respectively). A very strong positive relationship was reported between internal relatedness (IR) and Homozygosity by loci (HL), as Pearson's coefficient was estimated as 0.999, while between IR and H_{OBS} and between HL and H_{OBS} we observed strong negative correlations (−0.996 and −0.999, respectively). Regarding the subspecies *C. a.maximum*, similarly to *C. a. aspersum*, D had a positive relationship with H and d with Pearson's coefficient values of 0.861 and 0.770, respectively, while H also had a positive correlation with d (0.739). Moreover, it was reported that H_{OBS} had a strong negative relationship with *C. a. aspersum* HL (−0.901). On the contrary, in *C. a. maximum*, snails IR had a very strong

negative correlation with H_{OBS} with a Pearson's value of −0.917 and a very strong positive correlation between IR and HL (0.993). Finally, we observed that the heterozygosity of locus Ha5 had a strong relationship with the biotic load and the expected heterozygosity of Ha6 had a strong correlation with total rain during the production period (Pearson's 0.827 and 0.855, respectively).

Table 5. Genetic variation at each locus for each population of *C. a. aspersum* and *C. a. maximum*. Heterozygosity expected (H_{EXP}), Heterozygosity observed (H_{OBS}), Number of alleles (N_O), and F_{IS} values per population for each locus are reported. * HWE indicates the deviation from Hardy–Weinberg equilibrium. Statistically significant F_{IS} values are indicated in bold.

		Population									
		C. a. aspersum			***C. a. maximum***						
Locus		**KNT1**	**OMR**	**All**	**SDN**	**SRT**	**KNT2**	**VOL**	**AGR**	**VSL**	**All**
Ha5	**H_{EXP}**	0.885	0.856	0.882	0.922	0.891	0.924	0.891	0.877	0.876	0.904
	H_{OBS}	0.450	0.400	0.425	0.500	0.500	0.300	0.550	0.706	0.650	0.528
	No	11	12	14	13	10	11	11	13	10	16
	F_{IS}	**0.498**	**0.539**	0.518	**0.464**	**0.445**	**0.681**	**0.389**	**0.200**	**0.263**	0.416
Ha6	**H_{EXP}**	0.894	0.958	0.933	0.913	0.858	0.904	0.908	0.919	0.869	0.900
	H_{OBS}	0.550	0.750	0.650	0.800	0.800	0.500	0.700	0.790	0.550	0.690
	No	16	19	23	14	13	13	13	15	14	27
	F_{IS}	**0.391**	**0.221**	0.303	**0.126**	0.069	**0.453**	**0.233**	**0.144**	**0.373**	0.234
Ha8	**H_{EXP}**	0.905	0.838	0.879	0.847	0.889	0.924	0.945	0.916	0.900	0.912
	H_{OBS}	0.474	0.700	0.587	0.650	0.500	0.500	0.700	0.684	0.550	0.597
	No	14	14	21	13	16	15	17	14	13	25
	F_{IS}	0.483	**0.169**	0.333	**0.238**	**0.444**	**0.466**	**0.264**	**0.258**	**0.395**	0.345
Ha9	**H_{EXP}**	0.684	0.878	0.786	0.815	0.746	0.845	0.810	0.809	0.763	0.806
	H_{OBS}	0.632	0.550	0.587	0.632	0.750	0.450	0.650	0.579	0.500	0.596
	No	10	10	14	11	10	10	10	10	9	18
	F_{IS}	**0.079**	**0.380**	0.249	**0.230**	−0.005	**0.474**	**0.202**	**0.290**	**0.350**	0.260
Ha10	**H_{EXP}**	0.928	0.923	0.934	0.935	0.941	0.921	0.939	0.956	0.921	0.947
	H_{OBS}	0.450	0.800	0.625	0.632	0.500	0.450	0.400	0.526	0.500	0.501
	No	15	14	20	16	16	12	16	18	13	23
	F_{IS}	**0.522**	**0.136**	0.331	**0.330**	**0.475**	**0.518**	**0.580**	**0.456**	**0.463**	0.471
Ha11	**H_{EXP}**	0.921	0.928	0.933	0.937	0.955	0.941	0.928	0.946	0.941	0.950
	H_{OBS}	0.550	0.650	0.600	0.667	0.579	0.700	0.526	0.611	0.700	0.631
	No	16	14	20	15	17	16	14	15	16	27
	F_{IS}	**0.409**	**0.305**	0.357	**0.294**	**0.400**	**0.261**	**0.439**	**0.361**	**0.261**	0.336
All loci	**H_{EXP}**	0.869	0.897		0.895	0.880	0.910	0.903	0.904	0.878	
	H_{OBS}	0.518	0.642		0.647	0.605	0.483	0.588	0.649	0.580	
	HWE	*	*		*	*	*	*	*	*	
	No	13.67	13.83		13.67	13.50	13	13.50	14.17	12.50	
	F_{IS}	0.411	0.290		0.281	0.318	0.475	0.355	0.293	0.351	

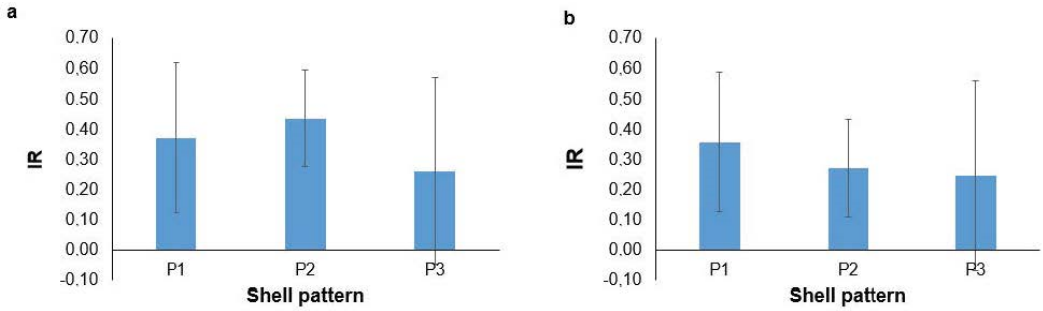

Figure 4. Shell patterns (P1, P2, and P3) and internal relatedness (IR) for (**a**) *C. a. aspersum* and (**b**) *C. a. maximum* populations.

4. Discussion

The current study indicated the morphological and genetic variation of populations of the snail subspecies of the dominant commercial species in Greece. Food authenticity is a crucial parameter of product quality and there is a lack of data concerning the genetic profile of farmed species such as the subspecies of the *Cornu aspersum*, *C. a. aspersum*, and *C. a. maximum*, which are the main farmed species in Greece [52]. The abundance of microsatellite gene sites, the high degree of polymorphism of microsatellite DNA, and many technical advantages make it a valuable molecular tool for demographic studies and studies of genetic structure of populations. Genetic diversity is the basis of ecosystem diversity and species diversity and each species has its own unique gene pool or form of genetic organization. To date, there have been many studies on the genetic diversity of species, including plants, insects, fish, and marine mollusks. Molecular polymorphism has not been studied in farmed snails. In this study, genetic variation was examined in six genetic loci: Ha5, Ha6, Ha8, Ha9, Ha10, and Ha11 [10].

The snail farms used for the sampling procedure were based in different localities and regions of Greece and a wide assortment of climatic types was indicated. Additionally, the open field farms were more vulnerable to local climatic conditions and low temperatures led to shorter production periods [53]. Controlled conditions during the production period lead to optimum growth rates in farmed snails compared with the wild ones. The age of collected snails ranged from 126 to 210 days. The production procedure of samples of both subspecies was similar, as the farms were equipped with low-pressure cooling systems and followed a feeding program mainly based on a compound diet. In our study, most farms used snails from other farms as farm generators and in the production period when sampling took place, snails of marketable size from the previous period were used as generators. Snail fillet, which is the main edible part of snails, has a commercial value and can be used an indicator of productivity, though it is not analyzed in depth. Differences in size, weight, and the morphometrical characteristics of snail shells can be explained by the species, the age of snails, the period of collection, the breeding conditions, and the diet for farmed species. Although the association between habitat use and relative shell height is consistent among species, the associations between habitat use and other shell variables (aperture diameter) differ among species in Hirasea snails [54]. After the F5 × F5 generation, the final size of the snails decreased [55]. In our study, all populations of *C. a. maximum* snails were bigger in size and weight than the populations of *C. a. aspersum* [24]. Farmed and wild *C. a. aspersum* snails had no significant differences in terms of weight and shell morphometrics [24]. In the current study, PCA revealed that the amount of variation of shell morphometrical characteristics and mass explained by each axis was 63.5% for the First Component and 12.2% for the Second Component in the case of *C. a. aspersum* populations and 73.7% for the First Component and 11.8% for the Second Component in the case of *C. a. maximum* populations.

We conducted the analysis of morphological characteristics and the grouping in three shell patterns was confirmed by the three resulting clusters after molecular analysis. More specifically, the population structure analysis revealed the existence of three haplotype clus-

ters among the population of the two farmed subspecies, *C. a. aspersum* and *C. a. maximum*, in Greece. In all the populations of the aforementioned subspecies, the coexistence of haplotypes from the three clusters highlighted the common gene pool not only for the populations from Kondariotissa, Sidini, and Souroti, which derived from snail farms S1, S2, and S3 belonging to the same snail farmer's cooperative. Martsikalis et al. [56] assessed the genetic variation of 10 populations of farmed trout in Greece and they found that samples derived from the common gene pool. The two monophyletic populations of Egyptian and Saudi Arabian *Eobania vermiculata* were found to represent two distinct groups, leading to the conclsusion that the two separate groups could be considered two separate subspecies [20]. Morphology is usually a feature associated with fitness and is therefore limited to strong selective pressures. The morphology of the gastropod shell is one of the most frequently studied morphological features due to its importance for taxonomic and evolutionary studies [17,56]. The snail shell color is diverse. In *C. a. aspersum* the shell can have three basic colors, yellow, red and brown, each of which is due to an allele of site C. Heterozygotes are usually intermediate in color. In this study, most snails of each population of both farmed subspecies, *C. a. aspersum* and *C. a. maximum*, belonged to shell pattern characterized by a light-colored shell with bands. Only, *C. a. aspersum* snails form Omorphochori and *C. a. maximum* snails from Agrinio did not have light-colored shells without bands. Additionally, the *C. a. maximum* populations which were collected from farms located at Sidini, Souroti, Alli Meria, and Vasileoniko did not have dark-colored shells. As reported in wild species *Cepaea hortensis* and *Theba pisana* and the farmed subspecies *C. a. maximum*, darker shells were observed in habitats with colder climates, while the lighter shells were observed in habitats with warmer climates [57]. The biotic load of the snail farm and the fact that farmed snails were used as ancestral populations most times without renewals might lead to breeding depression and similar morphologies. The snail mantle of farmed snails is another anatomical feature which was examined in the frame of polymorphism analysis. Mantle color presents polymorphism due to Mendelian inheritance at single loci, such as the heavily dark-spotted versus pale mantle in *Trichia striolata* (Pfeiffer) (Hyromiidae) [22]. *C. a. aspersum* snails were bred in Kondariotissa, with exception to the species *C. a. maximum*; a percentage of *C. a. aspersum* snails had an intermediate-colored mantle instead of a light-colored one. *C. a. maximum* shells were light-colored in snails that came from a farm where only this species was bred. *C. a. maximum* snails were from Vasileoniko, which was the only sea area of this study and where, in the past, *C. a. aspersum* snails were bred; these snails presented a small percentage of light-colored mantle. The local adaptation model generates predictable patterns [58]. As the environment of all farms did not present differences, local adaptation sets predictable outcomes regardless of whether a phenotype is the result of plastic or genetic adaptation.

In natural populations, environmental conditions are variable and this can affect the evolution of phenotypic plasticity [59]. In the same way that inbreeding can affect fitness and fitness-related traits, it may also affect trait plasticity [60]. Inbreeding may directly affect plasticity by altering phenotypic expression in one environment or by altering the organism's ability to detect or respond appropriately to different environmental conditions. Heterozygosity and population size should be positively correlated with fitness among populations of a species. It was reported that commonly used surrogates for fitness—heterozygosity, population size, and quantitative genetic variation—were positively and significantly correlated with population fitness but they explained only 15–20% of the variation in fitness [61]. We collected samples from F4–F13 generations except for KNT1 snails, which were an F1 generation for the farm. Most snail farms chose generators from other farms; the exact generation of our samples is unknown. According to the theory, low heterozygous individuals have a relative reduced fitness, possibly due to inbreeding depression [62]. The rate at which molecular heterozygosity is lost per generation (1/2 of the effective population size) also applies to the loss of additive genetic variation [63]. As reported in the last study [63], the rate at which molecular heterozygosity is lost per generation (1/2 of the true population size) also applies to the loss of additive genetic

diversity. A positive correlation was observed between the generation of snails used for the analyses and the H_{OBS} of the Hα10 genetic locus and the H_{EXP} of Hα9. In contrast, the age of snails of both subspecies did not show a correlation with heterozygosity at the tested genetic loci. HWE deviations are expected because of the gene pool.

Moreover, the level of differentiation between *C. a. aspersum* populations was significant while F_{ST} among *C. a. maximum* populations ranged from −0.0009 to 0.095 and no significant genetic differentiation was reported. The F_{ST} indicated between the two populations *C. a. aspersum* was 0.034, a value between the 0.019 and 0.634 observed in pairs of populations of *Murella muralis* [64]. The genetic differentiation among populations of both subspecies used in our research measured by the Fst value is generally described as negligible according to the categories for cultivated populations: negligible (0–0.05), moderate (0.05–0.15), great (0.15–0.25), and very great (above 0.25) [65].

In our data, for both farmed subspecies the observed heterozygosity of all populations of both species was lower than the expected due to the majority of homozygous individuals, as was also reported in populations of farmed trout in Greece [56]. Lower values of observed heterozygosity than the expected heterozygosity were also reported after molecular analysis of five microsatellite loci of the Helicidae species *Arianta arbustorum* [66]. In the aforementioned study, the observed heterozygosity had a value of zero for three loci. The study in land snail *M. muralis* revealed that almost all populations had a mean number of alleles greater than three [64]. As was reported by Arnaud et al. [67], microsatellite loci of *C. a. aspersum* snails belonging to the same colony were highly polymorphic with an overall total of 114 alleles, number of alleles per locus ranging from 5 (Ha2) to 32 (Ha13) while in our study *C. a. aspersum* populations were less polymorphic and presented from 14 alleles (Ha5) to 23 alleles (Ha6). Although snails of the aforementioned subspecies derived from farm in Omorphochori and generators that were renewed four times, the number of alleles for each locus was almost the same as snails from Kondariotissa. The aforementioned values of alleles were reported to snails derived from a farm where the only farmed species was *C. a. aspersum*. As was illustrated by mean values of alleles for all loci of each population *C. a. maximum*, these loci were more polymorphic.

In our study, farmed *C. a. aspersum* snails had no significant differences in weight and shell morphometric characteristics. In the case of subspecies *C. a. maximum*, snail mass, fillet mass and viscera mass did not show a normal distribution. In the present study, Principal Component Analysis (PCA) revealed that the amount of variation in shell morphometric characteristics and mass explained by each axis was 63.5% for the First Component and 12.2% for the Second Component in the case of *C. a. aspersum* populations and 73.7% for the First Component and 11.8% for the Second Component in the case of *C. a. maximum* populations. When the morphometric characteristics of the shell and masses of both subspecies were studied, a variation was observed with the First Component, showing a variability of 86.7%.

Developing a founder stock of the economically important species that have the genetic ability to use feed and resources efficiently [68] is not only crucial in aquaculture, but also in land species. Authenticity depends on the genetic background [38] and both morphological and molecular markers can reveal mislabeled food products such as aquatic products with uncommon trade names [69,70].

5. Conclusions

The current study comprises the first attempt to assess the morphological and genetic variation among and within farmed snail populations throughout Greece. The farmed populations of both subspecies presented three shell patterns. The percentage of genetic variation within populations reflected the admixture of initial populations, highlighting a common gene pool broodstock for snail farms throughout Greece.

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Institutional Review Board Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

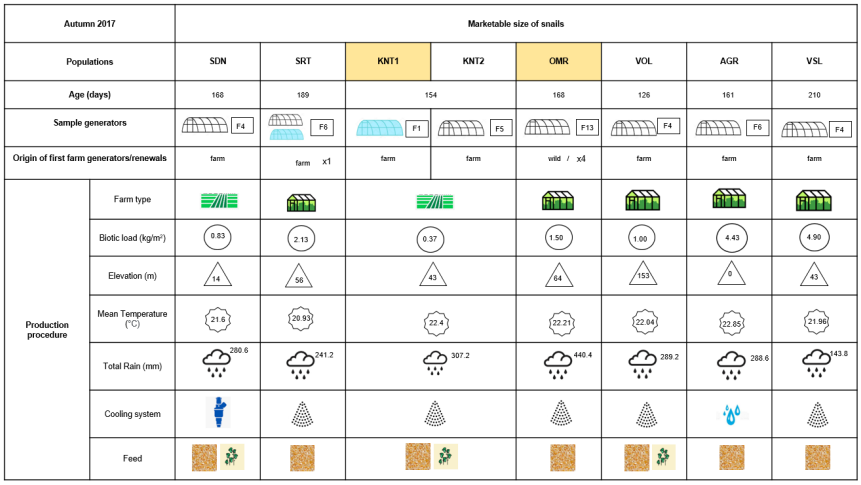

Figure A1. Breeding conditions and generators of snail populations until their collection. Yellow color is used for *C. a. aspersum* populations. For each population: age (days), origin (same farm/another farm) and generation of sample generators, origin of first farm generators (farm/wild) and times of renewals, type of farm (open filed/net covered greenhouse), biotic load, mean temperature and total rain during the production period, cooling system and feed (compound diet/and plants).

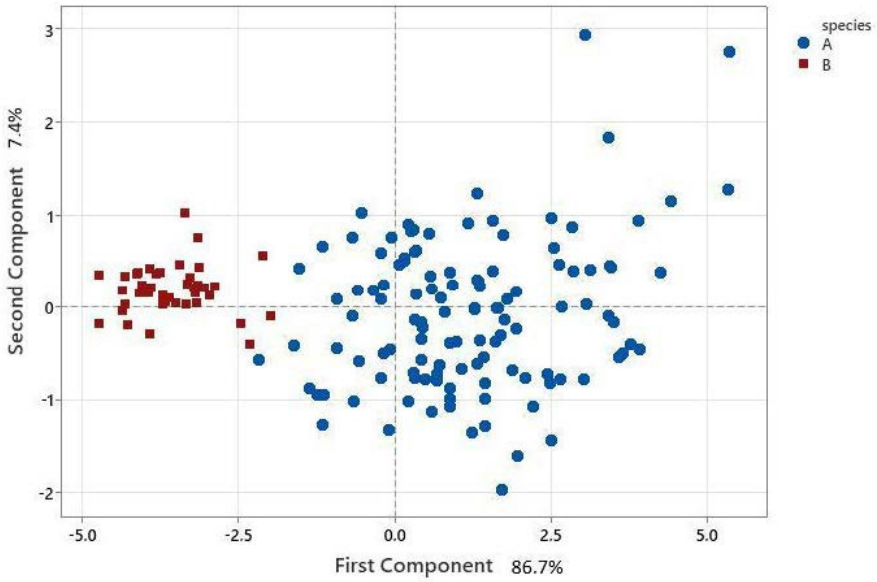

Figure A2. Differentiation of morphometrical characteristics among farmed populations of the subspecies *C. a. aspersum* (species A) and farmed populations of the subspecies *C. a. maximum* (species B) using Principal Component Analysis (PCA) ordination plot. The amount of variation explained by each axis is 86.7% for the First Component and 7.4% for the Second Component.

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