Chapter

A Comparison of Genetic Variation in Two Endemic Thermal Spring Isopods, *Thermosphaeroma thermophilum* and *T. milleri* (Crustacea - Isopoda: Sphaeromatidae)

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Abstract

Populations with reduced gene flow and restricted population size are expected to show reduced genetic variation. Using starch gel electrophoresis, we examined allozyme variation at 12 loci in two species of freshwater, sphaeromatid isopods. *Thermosphaeroma thermophilum*, an endangered species, inhabits a single thermal spring in central New Mexico, USA; and *T. milleri*, inhabits a more complex thermal spring system in northern Chihuahua, México. We found no significant differences in allelic variation between the sexes within each species. Between species, electromorphs at each locus differed significantly in both number and moiety on the gel, with *T. milleri* showing greater polymorphism and greater heterozygosity than *T. thermophilum*. Nei's unbiased genetic distance, calculated using the nine loci common to both populations (D = 0.75), was consistent with morphological classification of *T. thermophilum* and *T. milleri* as separate species, as well as with molecular analyses suggesting that these populations have been separated since the late Cretaceous (88 myr). Moreover, consistent with the theoretical expectation that small, isolated populations will exhibit reduced genetic variation, *T. thermophilum*, an endangered species, exhibited significantly less genetic variation than the more numerous and less confined *T. milleri*. We compare our results with other recent studies using this approach to understand the population genetics of natural populations.

Keywords: allozymes, endangered species, Mexico, New Mexico, Socorro isopod

1. Introduction

Wright [1] observed that restricted gene flow and reduced population size can lead to population differentiation and ultimately to speciation. Genetic isolation by distance as well as speciation occurring in the presence of geographic barriers (i.e., allopatric speciation) are well-documented phenomena in a range of taxa [2–4].

Genetic variation is expected to decrease with decreased population size due to fixation of alleles. Frankham [5] empirically demonstrated this effect by comparing genetic variation of island populations and their mainland counterparts. A significant majority of island populations showed reduced genetic variation (average reduction in heterozygosity = 29%, N = 202 comparisons). Moreover, insular endemic species showed significantly greater reductions in genetic variation compared to non-endemic populations [6]. Reduced genetic variation has a strong correlation with reduced population fitness [6]. Species that occupy particular freshwater habitats often have limited abilities to disperse to alternative environments [7, 8]. Freshwater endemic populations are therefore extremely vulnerable to extinction, and a detailed understanding of their genetic diversity is valuable to conservation efforts [6].

With increasing sophistication of methods for assessing genetic variation and documenting parentage using DNA sequence data [9], starch gel electrophoresis has been displaced in favor of these demonstrably more precise methods. Nevertheless, allozyme analysis retains certain advantages over molecular methods; it is cheaper, involves fewer procedural steps, seldom requires optimization, and requires no exploratory studies to identify primers [10]. Most importantly, genetic differences identified by using allozymes provide a clear and conservative method for distinguishing populations. Thus, while no longer fashionable, starch gel electrophoresis remains a useful approach for estimating genetic variation within and among species. Recent studies confirm the continued value of the starch gel and other protein electrophoresis methods for investigating population differentiation across a broad range of taxa, including plants [11–14], invertebrates [15–17] and vertebrates [18–20].

The sphaeromatid isopod genus, *Thermosphaeroma*, consists of seven known species, each endemic to a single, thermal, freshwater habitat in southwestern North America. Monophyly within this genus is unambiguous [21], thus *Thermosphaeroma* provides an excellent system for genetic and evolutionary comparisons among species. In this paper, we report genetic variation in two species of *Thermosphaeroma*; *T. thermophilum*, the endangered Socorro isopod [22, 23] is endemic to a single thermal spring near Socorro, New Mexico, USA. Its congener, *T. milleri*, is also an endemic species, but inhabits a larger and spatially more complex spring system located west of Villa Ahumada, Chihuahua, México, and has a greater estimated population size (Shuster, unpubl. data). Due to the large geographic distance separating these two endemic species (over 500 km), and thus the extreme unlikelihood of gene flow between populations, we expected genetic differentiation between these species to be considerable, despite their close taxonomic relationship [21]. We also expected genetic variation to be significantly reduced in *T. thermophilum* compared to *T. milleri*, given the above predictions of population genetic theory as well as with the Socorro isopod's status as an endangered species [24, 25]. As this is the first detailed description of allozyme variation in this genus, we provide descriptions of electromorphs at each locus. We know of no studies comparing genetic variation between closely related, endemic species with such marked differences in habitat and population size.

2. Materials and methods

2.1 Sampling and processing of isopods

T. thermophilum: Sedillo Spring, the natural habitat of *T. thermophilum*, was modified in the early 1900s to supply water to a thermal spring bathhouse (Evergreen) as well as to the city of Socorro, New Mexico [26]. The bathhouse is now abandoned,

and the species is confined to the concrete pools and gutters through which the remaining surface waters of the spring flow. More detailed descriptions of this restricted habitat and estimates of population size (<3000 individuals) are provided in Federal Register [22] and Shuster [23–25, 27–30].

Using a fine mesh net, we collected samples of approximately 100 *T. thermophilum* from the substrate and walls of pool 2 [23] in November 1993 and in June 1995. In 1993, live isopods were placed in insulated containers with spring water and transported to Flagstaff, AZ, where 13 adult males and 11 adult females from the original sample were placed on ice for 10 min, euthanized by removing the cephalon with sharp forceps, and divided into five tissue sub-samples (cephalon, upper pereon, lower pereon, pereopods and pleotelson). Each tissue sub-sample was placed into a well in one of five separate ELISA dishes with 25 l 0.05 M Tris-HCl buffer and frozen at −80°C until samples were electrophoresed. In 1995, isopods were transported to Flagstaff for use in behavioral experiments [28–30]. Tissue samples were collected from live specimens by placing individuals on ice for 10 min and then amputating and freezing the left or right 7th pereopod.

T. milleri: Several thermal springs exist on Ejido Rancho Nuevo, located west of Villa Ahumada in northern Chihuahua, México. The largest spring, Ojo Caliente, arises from at least five sources within a 200 m^2 area beneath a large cottonwood tree and flows SSW to form a 3–5 m wide stream, which is diverted to supply water to Ejido crops and livestock. Mosquitofish, pupfish, crawfish and large planarians all inhabit the stream whose gravel and mud banks are well-covered with grass and other aquatic vegetation. The population size is estimated to exceed 1.5×10^5 individuals (Shuster, unpubl. data). More details of this habitat are provided in Bowman [31] and Davis [21].

Using a fine mesh net, we collected approximately 100 *T. milleri* from the substrate and vegetation of Ojo Caliente in August 1994 and in December 1995. The 1994 sample was processed as described above for *T. thermophilum*. The 1995 sample was maintained in laboratory aquaria for use in behavioral experiments [28–30]. Tissue samples were collected from live specimens by placing individuals on ice for 10 min and then removing the left or right seventh pereopod with fine forceps. Each pereopod was frozen as described above until samples were electrophoresed.

2.2 Electrophoresis

Frozen tissue samples were thawed on crushed ice, ground with an additional 30 ml 0.05 M Tris-HCl pH 7.5 buffer using a glass rod, and loaded onto 12% starch gels using buffer systems described in Sassaman [32]. Gels were run at 35 mA for 4 h, sliced and stained for 12 enzyme loci (see below). Electrophoretic signals were identified by measuring the height of each signal's leading edge on the gel above its sample well on the gel, and were scored among the two *Thermosphaeroma* species by identifying the fastest running electromorph as "1" and slower electromorphs as 2–3. To verify the relative position of electromorphs on gels between species after all individuals were initially scored, we reran samples from individuals who exhibited the range of allelic variation for both species on the same gels.

For each locus and within each sex, sample and species, we identified three measures of allelic diversity, the polymorphic index [PI = $1 - (\Sigma p_i^2)$, where p_i = the population frequency of each allele, i], the effective number of alleles [ENA = $1/(\Sigma p_i^2)$], and observed heterozygosity (H). We also performed goodness of fit G-tests for deviations from Hardy-Weinberg (hereafter HW) expectations [4]. We calculated the average and standard error of the first three measures and compared PI, ENA and H across loci using U-tests, first between sexes within species using all samples collected, then between species with the sexes pooled. Because of the large

geographic distance separating these two species (>500 km), we did not estimate Wright's F-statistics, assuming that gene flow between these morphologically distinct populations was negligible. Instead we calculated [33] unbiased genetic distance (D) using used Miller's [34] TFPGA which provides a weighted average estimate of D across all loci. We assumed the inbreeding coefficient within each collection equaled F = (1—H) [4].

3. Results

3.1 Electrophoretic signals

The two *Thermosphaeroma* species exhibited two groups of electrophoretic signals, those in which alleles appeared to be similar in moiety on the gel between species (that is, electromorphs appears to reside at similar positions with respect to one another on the gel), and those in which alleles appeared distinct in both moiety and character. We observed apparently similar electromorphs between the species at *Pgm*1, *Pgi*, *Me*, *Mdh*1, *Mdh*2 and 6*pgdh*. Distinct electrophoretic signals appeared at *Pgm*2, *Got*, *Xdh*, *Idh*1, *Idh*2 and *Hex*.

In *T. thermophilum*, phosphoglugomutase loci (*Pgm*1 and *Pgm*2) were monomorphic, visible as tight bands, located at 31.7 and 28.8 mm, respectively, above sample wells. In *T. milleri*, only *Pgm*1 was visible. This locus also appeared as a tight band but exhibited three alleles. $Pgm1^1$ appeared identical in both species, with alleles $Pgm1^2$ and $Pgm1^3$ in *T. milleri* visible at 27.1 and 22.7 mm, respectively, above sample wells.

Phosphoglucose isomerase (*Pgi*) appeared equivalent in moiety in both species, with signals visible as tight bands with small amounts of trailing anodic signal. Two alleles were identifiable, with Pgi^1 located 21.6 mm, and Pgi^2 located 18.9 mm, above sample wells. In both species, heterozygotes appeared as trimers, suggesting that the functional *Pgi* molecule consists of two units which combine at random [35].

Both species shared a similar fast signal at malic enzyme (Me^1), located at 20.2 mm above sample wells; *T. thermophilum* was monomorphic for this allele, whereas *T. milleri* showed two additional loci, Me^2 at 16.4 mm and Me^3 at 14.9 mm above sample wells. All signals in both species appeared as tight, dense bands. At 6-phosphoglugose dehydrogenase (6*pgdh*), the fast electromorph (6-$pgdh^1$) and the intermediate electromorph (6-$pgdh^2$) appeared similar for both species at 37.9 and 32.5 mm above sample wells, respectively. *T. milleri* showed a third electromorph (6-$pgdh^3$) at 29.8 mm, which was not observed in *T. thermophilum*.

Malic dehydrogenase 1 (*Mdh*1) has been recognized as a mitochondrial signal in certain crustaceans (C. Sassaman, pers. com.), thus recognition of alleles at this locus is questionable. However, we identified similar fast signals in both species ("$Mdh1^1$"), appearing as broad bands at 35.5 mm above sample wells, as well as a slower signal ("$Mdh1^2$") in *T. milleri* appearing at 30.4 mm above sample wells. Although we did not include this locus in HW analyses or in calculations of D, we have provided estimates of PI and ENA to illustrate the diversity in these signals within each species. The fast electromorph at *Mdh*2 reached similar locations above sample wells in both species ($Mdh2^1$ at 10.7 mm). However, signal density differed such that in the monomorphic *T. thermophilum* $Mdh2^1$ signal was clearer and more narrow, whereas in *T. milleri* signals were polymorphic ($Mdh2^2$ at 5.1 mm), less dense and showed considerable trailing anodic signal.

The species were distinct at glutamate oxaloacetate transaminase (*Got*). A single allele was visible in *T. thermophilum* as a tight band 38.7 mm (Got^1) above sample wells. In *T. milleri*, two alleles were visible as somewhat broader bands at 29.4 mm (Got^1) and 24.9 mm (Got^2) above sample wells. The species were distinct at xantine

dehydrogenase (*Xdh*) as well. Both species showed broad, monomorphic signals; *T. thermophilum* at 12.0 mm (Xdh^1) and *T. milleri* at 18.3 mm (Xdh^2). Species differences were also clear at hexokinase (*Hex*), both species showing broad and variable, but distinct signals. Two alleles were visible in *T. thermophilum*, Hex^1 at 33.4 mm and Hex^2 at 24.6 mm, and three alleles were visible in *T. milleri*, Hex^3 at 23.2 mm, Hex^4 at 19.8 mm and Hex^5 at 12.8 mm.

3.2 Genetic differences between species

Allele frequencies at all loci in both species conformed to Hardy-Weinberg equilibrium (**Table 1**), suggesting that Mendelian inheritance and selective neutrality can be assumed for these loci [36]. We found no significant differences in polymorphic indices (PI), effective numbers of alleles (ENA) or heterozygosity (H) between the sexes within either species (U-tests: *T. thermophilum*: PI (mean ± SE, n = 17): males, 0.06 ± 0.04; females, 0.08 ± 0.05; $z = -0.22$, $P = 0.83$, NS; ENA: males, 1.09 ± 0.07; females, 1.13 ± 0.09; $z = -0.22$, $P = 0.83$, NS; H: males, 0.05 ± 0.03; females, 0.22 ± 0.14; $z = -0.18$, $P = 0.86$, NS; *T. milleri*: PI (mean ± SE, n = 10): males, 0.20 ± 0.06; females, 0.29 ± 0.10; $z = -0.31$, $P = 0.76$, NS; ENA: males, 1.33 ± 0.12; females, 1.23 ± 0.19; $z = -0.38$, $P = 0.70$, NS; H: males, 0.22 ± 0.06; females, 0.30 ± 0.13; $z = -0.19$, $P = 0.85$, NS). Therefore, the sexes were combined for further analysis.

Locus	Species	Sample	Sex	N	EMs	PI	ENA	H	HW	P
PGM1	*thermophilum*	11/93	M	13	1	0.00	1.00	0.00	—	—
			F	12	1	0.00	1.00	0.00	—	—
		6/94	M	100	1	0.00	1.00	0.00	—	—
			F	14	1	0.00	1.00	0.00	—	—
	milleri	12/95	M	103	3	0.50	1.99	0.52	0.28	N.S.
PGM2	*thermophilum*	11/93	M	13	1	0.00	1.00	0.00	—	—
			F	12	1	0.00	1.00	0.00	—	—
		6/94	M	100	1	0.00	1.00	0.00	—	—
			F	14	1	0.00	1.00	0.00	—	—
	milleri	12/95	No signal							
GOT	*thermophilum*	11/93	M	13	1	0.00	1.00	0.00	—	—
			F	12	1	0.00	1.00	0.00	—	—
	milleri	12/95	M	13	2	0.14	1.17	0.15	0.09	N.S.
			F	12	1	0.00	1.00	0.00	—	—
PGI	*thermphilum*	11/93	M	13	1	0.00	1.00	0.00	—	—
			F	12	1	0.00	1.00	0.00	—	—
		6/94	M	100	2	0.02	1.02	0.00	0.01	N.S.
			F	14	1	0.00	1.00	0.00	—	—
	milleri	12/95	M	103	3	0.25	1.34	0.25	0.52	N.S.
			F	12	2	0.08	1.09	0.08	0.02	N.S.
XDH	*thermophilum*	11/93	M	13	1	0.00	1.00	0.00	—	—
			F	12	1	0.00	1.00	0.00	—	—
	milleri	12/95	M	4	1	0.00	1.00	0.00	—	—
			F	6	1	0.00	1.00	0.00	—	—
ME	*thermophilum*	11/93	M	13	1	0.00	1.00	0.00	—	—

Locus	Species	Sample	Sex	N	EMs	PI	ENA	H	HW	P
			F	12	1	0.00	1.00	0.00	—	—
		6/94	M	75	1	0.00	1.00	0.00	—	—
			F	5	1	0.00	1.00	0.00	—	—
	milleri	12/95	M	35	3	0.27	1.37	0.31	1.22	N.S.
			F	12	2	0.15	1.18	0.20	0.10	N.S.
IDH1	*thermophilum*	11/93	M	9	1	0.00	1.00	0.00	—	—
			F	5	1	0.00	1.00	0.00	—	—
	milleri	12/95	M	13	1	0.00	1.00	0.00	—	—
IDH2	*thermophilum*	11/93	M	13	1	0.00	1.00	0.00	—	—
			F	12	1	0.00	1.00	0.00	—	—
	milleri	12/95	No signal							
MDH1	*thermophilum*	11/93	M	13	1	0.00	1.00	0.00	—	—
			F	12	1	0.00	1.00	0.00	—	—
		6/96	M	85	2	0.12	1.14	0.13	0.41	N.S.
			F	9	2	0.20	1.24	0.22	0.14	N.S.
	milleri	12/95	M	102	2	0.13	1.15	0.14	0.55	N.S.
			F	11	1	0.00	1.00	0.00	—	—
MDH2	*thermophilum*	11/93	No signal							
	milleri	12/95	M	103	2	0.07	1.08	0.08	0.17	N.S.
			F	11	1	0.00	1.00	0.00	—	—
HEX1	*thermophilum*	11/93	M	13	2	0.43	1.75	0.31	1.00	N.S.
			F	8	2	0.50	2.00	0.75	2.00	N.S.
	milleri	12/95	M	13	3	0.50	2.00	0.55	3.57	N.S.
			F	4	2	0.43	1.75	0.43	0.29	N.S.
6PGDH	*thermophilum*	11/93	M	13	1	0.00	1.00	0.00	—	—
			F	12	1	0.00	1.00	0.00	—	—
		6/96	M	48	2	0.06	1.06	0.06	0.05	N.S.
			F	17	2	0.13	1.15	1.43	0.04	N.S.
	milleri	12/95	M	100	3	0.14	1.16	0.15	0.66	N.S.
			F	11	3	0.24	1.32	0.27	0.27	N.S.

Number of electrophoretic morphs (EMs), polymorphic index (PI), effective number of alleles (ENA), observed heterozygosity (H), results of G-test for deviation from the Hardy-Weinberg equilibrium (HW), and significance of deviation (P).

Table 1.
Electrophoretic variation in Thermosphaeroma thermophilum and T. milleri.

Between species, each of the three estimators of genetic variation differed significantly (U-tests, $n = 54$: PI: *T. thermophilum*: 0.07 ± 0.03, $n = 34$; *T. milleri*: 0.25 ± 0.06, $n = 20$; $z = -3.08$, $P = 0.002$; ENA: *T. thermophilum*: 1.11 ± 0.06, $n = 34$; *T. milleri*: 1.28 ± 0.11, $n = 20$; $z = -2.48$, $P = 0.01$; H: *T. thermophilum*: 0.13 ± 0.07, $n = 34$; *T. milleri*: 0.26 ± 0.07, $n = 20$; $z = -2.55$, $P = 0.01$). Nei's [33] unbiased genetic distance, D, equaled 0.75, a value consistent with species-level genetic differences in a number of species [33].

4. Discussion

Strong, directional selection could explain reduced genetic variation in *T. thermophilum* compared to *T. milleri*, although we found no significant deviation from HW expectations at the four variable loci (**Table 1**) in *T. thermophilum*, and no deviations from HW expectations at any locus examined in *T. milleri*. The *T. thermophilum* population is reported to have undergone a severe bottleneck in the mid-1980s, when a valve controlling the water supply to Sedillo Spring was destroyed by vandals (B. Lang, pers. comm.). This event, as well as past destruction of the natural habitat by private and municipal water projects may also have reduced the available habitat, reduced population size, and thus reduced genetic variability in the *T. thermophilum* population. This explanation is consistent with average heterozygosity (H) in *T. thermophilum* equaling half of that observed in *T. milleri*.

However, these factors do not explain why some loci (*Hex*[1]; 6-*pgdh*) in *T. thermophilum* have remained highly variable. Recent bottlenecks in other species appear to reduce genetic variation in all loci simultaneously [37, 38] and reductions in allelic diversity may persist for millennia [39]. Selection can maintain polymorphism in finite populations [4]. However, in Sedillo Spring, the source of selection and the isopod characteristics on which it may act, are unknown. Thus, the existence of genetic variation in some but not all loci in the endangered Socorro isopod population, while encouraging from a species management perspective, for now remains unexplained.

Genetic population structure is well-documented in isopod crustaceans using a variety of genetic markers. Allozymes are most commonly used to identify genetic differences within and among populations [36, 40–43] although pigmentation patterns known to exhibit Mendelian inheritance have been and are still widely used in documenting population differences [44–49]. Molecular markers are increasingly used to document genetic differences among isopod populations, and preliminary data using mitochondrial DNA sequences have been used successfully to distinguish congeners within the genus *Thermosphaeroma* [22].

Although molecular genetic methods do provide more precise information on genetic differences within and among populations, protein electrophoresis remains a useful tool for investigating population differences. Because allozyme loci are usually codominant, clear indications of allelic differentiation, isolation by distance and most standard measures of genetic diversity can all be identified without approximation [12–14, 16]. Population differences detected using this approach are certain to be more conservative than molecular analyses [9]. Moreover, despite continued reduction of the cost of molecular analyses, electrophoresis is remains a cheaper and more sample efficient method—particularly for large samples—than most analyses using DNA.

Our results using allozyme variation, indicate that *Thermosphaeroma milleri* and *T. thermophilum* are genetically distinct at the level of separate species, a result consistent with previous morphological [49] and molecular genetic analyses [22]. Our main finding, that genetic variation in *T. thermophilum* is sharply reduced compared to its closest relative, is consistent with population genetic theory [1, 3, 4] and with recent empirical analyses [5] indicating that reduced population size reduces genetic variation within populations, specifically within the *T. thermophilum* population.

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