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Genetic divergence in Northamerican freshwater planarians of the Dugesia dorotocephala group (Turbellaria, Tricladida, Paludicola)

By G. Nascetti, L. Bullini and M. Benazzi

Abstract

The genetic differentiation between the members of the *Dugesia (Girardia) dorotocephala* group was analyzed by means of multilocus electrophoresis, and compared to that of another planarian species, *D. tahitiensis*, also belonging to the subgenus *Girardia*. The species examined were: *D. dorotocephala* s.s (2n = 16), *D. arizonensis* (2n = 8), *D. jenkinsae* (2n = 8), and the above mentioned *D. tahitiensis* (2n = 16). The former three species inhabit North America, and show different proportion of fissiparous and sexual individuals; the latter species inhabits Polynesia and is fully asexual. A total of 11 enzyme loci were genetically analyzed: *Mdh-1, Mdh-2, Idh-1, Idh-2, G3pdh, Got-1, Ck, Pgm-2, Ada, Mpi*, and *Gpi*. Low values of observed mean heterozygosity per locus (*Ho*) were found in the populations studied, ranging from 0 to 0.18 (average 0.08). In asexual populations (except that of *D. tahitiensis*) fixed heterozygosity was observered in all the individuals for 1 or 2 loci. The genetic divergence between the species examined is very high, with many loci showing discriminating alleles in different taxa (Nei's genetic distance varies from 0.871 to 1.759). The populations of *D. dorotocephala* s.s., on the contrary, appear to be genetically quite homogenous (average *D* = 0.019), and the genetic distance values are apparently unrelated to their geographic location and to their way of reproduction. The genetic distance between *D. tahitiensis*, a species not included in the *D. dorotocephala* group, and *D. dorotocephala* s.s. is 1.314 and hence similar to the *D* value between two members of the *dorotocephala* group: *D. dorotocephala* and *D. jenkinsae* (*D* = 1.303). The genetic relationships among the populations studied were established by UPGMA cluster analysis and multidimensional scaling. The descendence of the North American species with 2n = 8 from a *dorotocephala*-like ancestor with 2n = 16 is considered. It is suggested that the latter, as well as a *tahitiensis*-like line, also having 2n = 16, have originated from a common ancestor by geographic isolation.

Key words: Freshwater planarians – *Dugesia dorotocephala* – Multilocus electrophoresis – Genetic divergence – Evolutionary relationships

Introduction

Freshwater planarians provide an excellent experimental material for various biological studies, due to their extremely high regenerative power and to their capability of asexual reproduction (fissioning). Asexual reproduction shows different patterns, according to the species, race, or strain: fissioning may be constant, producing clones in which normal sexuality never takes place, or it may regularly switch to sexual reproduction.

The ability for fissioning, although largely influenced by environmental factors, seems to be caused by a single gene mutation (Benazzi 1974).

Polyploidy and aneuploidy occur frequently in planarians. In some cases intraspecific differentiation of biotypes (either diploid or polyploid with complex cytological mechanisms) has been described (for a review see Benazzi and Benazzi-Lentati 1976).

Most experimental and karyological investigations were carried out on the genus *Dugesia*, which comprises three subgenera: *Dugesia* and *Schmidtea* from the Old World, and *Girardia* mainly from the New World. Particularly interesting is the case of *Dugesia*

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(Girardia) dorotocephala (WOODWORTH 1897). This freshwater flatworm is largerly distributed in North America, from Southern Canada to Northern Mexico. It was regarded as a polytypic species, in which either sexual or fissiparous reproduction prevails in the different populations. Karyological investigations were carried out by one of us (BENAZZI 1966, 1975) on populations of this planarian from various U.S. locations kindly provided by Dr. MARY M. JENKINS (at that time professor at Madison College, Harrisonburg, Virginia). A karyotype with 2n = 16 was found for most of these populations; only two of them, from San Felipe (Texas) and from Sabino (Arizona) respectively, showed a similar karyotype with a diploid complement of 8 chromosomes. The planarians from San Felipe are always sexual, very large, and have ventral testes like *D. dorotocephala*; they were described by BENAZZI and GOURBAULT (1977) as a distinct species: *Dugesia jenkinsae*. On the other hand, the planarians from Sabino have a long and thin body and reproduce constantly by fission. The population seems to be a fissiparous form of a third species: *D. arizonensis*, described by KENK (1975). This species reproduces sexually and resembles *D. dorotocephala* in its general appearance, but differs by having subdorsal instead of ventral testes. Moreover, its karyotype is 2n = 8, similar to that found in *D. jenkinsae* and in the fissiparous population from Sabino (GOURBAULT 1977a). *D. arizonensis* was found in two localities from Arizona: Bog springs in Madera Canyon (Santa Rita Mountains, Santa Cruz County) and Rucker Creak (Chirichuaua Mountains, Cochise County; both are not far from Sabino. The specimens from Rocker Creak appear more slender, resembling those from Sabino. Moreover, KENK(1975) reports that he has observed fissions sporadically in his laboratory cultures. BENAZZI (unpublished data) was able to induce partial sexuality in some specimens from Sabino, by feeding them for many weeks on crushed tissues of sexual planarians of different species (method by GRASSÒ and BENAZZI 1973). These specimens showed a few testicular follicles in a subdorsal position, as in *D. arizonensis*. The assignment of the population from Sabino to *D. arizonensis* appears therefore supported by evidence at different levels: kayrological, geographical and morphological (both external and internal).

The three species: *D. dorotocephala* s.s., *D. jenkinsae* and *D. arizonensis* are considered as members of the *D. dorotocephala* group, due to their low morphological differentiation. The phylogenetic relationships between these species were not yet clarified, as well as the relationships between the two karyotypes (2n = 8 and 2n = 16, respectively) found in these planarians.

The aim of this paper is to investigate the differentiation at gene level within the *D. dorotocephala* group by means of multilocus electrophoresis, an approach largely and successfully used in recent years in many animal and plant groups (e.g. see AVISE 1974; AYALA 1975; BULLINI and SBORDONI 1980; FERGUSON 1980). For comparison, a fourth species was analyzed; *D. tahitiensis* GOURBAULT (1977). This fissiparous form, with 2n = 16, is not included in the *dorotocephala* group, but belongs to the same subgenus *Girardia* (GOURBAULT 1977b).

Material and methods

The specimens studied electrophoretically were cultured by BENAZZI from material provided by Dr. M. M. JENKINS and Dr. I. KAUFMAN (*D. dorotocephala* s.l.) and by Dr. GOURBAULT (*D. tahitiensis*). The geographic origin, mode of reproduction and sample designation of the populations studied are as follows:

D. dorotocephala s.s. Suffern Mahewak river (New York); constantly asexual; SUF. – Penn State (Pennsylvania); mainly asexual, a sexual strain developed in the laboratory; PEN. – Blacksburg (southern Virginia); asexual, a few individuals became sexual in the laboratory; BLA. – Salado (Texas); several specimens became sexual in the laboratory; SAL.

D. jenkinsae. San Felipe (Texas); sexual, fissioning never observed; in laboratory culture the production of cocoons is extremely rare; SFE.

D. arizonensis. Sabino (Texas); constantly asexual; SAB.

D. tahitiensis. Tahiti Island (Polynesia); constantly asexual; TAH.

For the electrophoretic tests, homogenates were obtained by mechanically grinding single specimens in distilled water. Each homogenate was absorbed into 5 by 5 mm squares of chromatography paper (Whatman 3MM), and then inserted in 10 % starch gel. Standard horizontal electrophoresis was performed at 7–8 V/cm at 5 °C for 4–5 h. After the run, gels were cut into two slices and each part was stained for a specific enzyme. The following enzymes were studied: malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), glyceraldheyde-3-phosphate dehydrogenase (G3PDH), glutamate oxaloacetate transaminase (GOT), creatine kinase (CK), phosphoglucomutase (PGM), adenosine deaminase (ADA), mannose phosphate isomerase (MPI), and glucose phosphate isomerase (GPI). The buffer systems and staining techniques used are given in Tables 1 and 2.

Loci and alleles were designated as follows: isozymes were numbered in order of decreasing mobility from the most anodal; allozymes were named numerically according to their mobility relative to the commonest one in the reference population (*D. dorotocephala* s.s. from Salado, Texas), indicated as *100* (> *100* = faster mobility, < *100* = slower mobility).

A total of 11 loci were genetically analyzed: *Mdh-1, Mdh-2, Idh-1, Idh-2, G3pdh, Got-1, Ck, Pgm-2, Ada, Mpi,* and *Gpi.*

The amount of genetic divergence between populations was estimated using Nei's (1972) and Rogers' (1972) formulas. The genetic relationships among populations were represented on the basis of Nei's matrix of genetic distances using UPGMA cluster analysis (Sokal and Sneath 1963) and multidimensional scaling (Guttman 1968).

Table 1. **Buffer systems**
Analytical grade reagents per litre; pH at room temperature

Buffer system	Electrodes	Gel
1. Continuous Tris/citrate (Selander et al., 1971)	0.687 M Tris / 0.157 M citric acid, pH 8 (83.2 g Tris, 30 g monohydrate citric acid)	0.023 M Tris / 0.005 M citric acid, pH 8 (2.77 g Tris, 1.10 g monohydrate citric acid)
2. Tris/maleate (modified from (Brewer and Sing 1970)	0.01 M Tris / 0.1 M maleic acid / 0.01 M EDTA / 0.015 M $MgCl_2$ / 0.125 M NaOH, pH 7.2 (12.11 g Tris, 11.61 g maleic acid, 3.72 g EDTA, 2.03 g $MgCl_2$, 5 g NaOH)	electrode buffer diluted 1:10, pH 7.4
3. Tris/versene/ borate (Brewer and Sing 1970)	0.21 M Tris / 0.15 M boric acid / 0.006 M EDTA, pH 8 (25.4 g Tris, 9.24 g boric acid, 2.20 g EDTA)	0.021 M Tris / 0.02 M boric acid / 0.0007 M EDTA, pH 8 (2.5 g Tris, 1.24 g boric acid, 0.25 g EDTA)

Results and discussion

The allele frequencies found in the studied populations at the 11 loci analyzed are given in Table 3. The large majority of the loci appear to be monomorphic in the different populations at the electrophoretic level. Exceptions are the loci *Idh-1, Got-1,* and *Ck,* that are polymorphic in one or more samples. While sexual populations show Mendelian segregation at these loci, fixed heterozygosity was often found in the asexual populations. The values of observed mean heterozygosity per locus (*Ho*) ranged from 0 to 0.18, with an average of 0.08 (Table 3).

The values of Nei's and Rogers' genetic distance between the populations tested are given in Table 4. Nei's average genetic distance within *D. dorotocephala* s.s. is $D = 0.019$, a value often found in different animal groups among local populations of the same species. Also between populations geographically far apart and with different incidence of sexuality, such as Suffern (New York) and Salado (Texas), the genetic distance remains low ($D = 0.007$ in the above mentioned comparison).

The genetic divergence between the species examined is, on the contrary, very high, most loci showing distinct alleles differences between two or more taxa. No shared alleles

Table 2. **Electrophoretic procedures**

Enzyme	Buffer system	V/cm	Time	Staining buffer	Coenzymes	Linking enzymes	Substrates	Activators, inhibitors	Visualization methods
EC 1.1.1.37 MDH	1	8	5 h	0.05 M Tris/HCl pH 8 30 ml	NAD 15 mg		L-malic acid 1 M pH 7 5 ml		MTT 10 mg PMS 2 mg agar 0.8 %
EC 1.1.1.42 IDH	1	7	4 h	0.05 M Tris/HCl pH 8 30 ml	NADP 5 mg		DL-isocitrate 30 mg	$MgCl_2$ 10 mg	MTT 10 mg PMS 2 mg agar 0.8 %
EC 1.2.1.12 G3PDH	1	8	4 h	0.05 M Tris/HCl pH 8 50 ml	NAD 30 mg	EC 4.1.2.13 ALD 0.1 ml	fructose-1,6-diphosphate 125 mg incubate with ALD for 30′	sodium arse-nate 150 mg	MTT 10 mg PMS 2 mg
EC 2.6.1.1 GOT	1	7	4½ h	0.2 M Tris/HCl pH 8 50 ml			aspartic acid 200 mg, α-chetoglutaric acid 100 mg; adjust to pH 7.5 with 1 M Tris. Pour on gel, incubate for ½ h then add Fast Blue BB	pyrydossal-5′-phosphate 10 mg	Fast Blue BB 150 mg
EC 2.7.3.2 CK	1	7	4 h	0.05 M Tris/HCl pH 8 30 ml	NADP 5 mg	EC 1.1.1.49 G6PDH 0.02 ml EC 2.7.1.1 HK 0.02 ml	ADP 20 mg, creatine phosphate 180 mg, glucose 250 mg	$MgCl_2$ 10 mg	MTT 10 mg PMS 2 mg agar 0.8 %
EC 2.7.5.1 PGM	2	7	4 h	0.05 M Tris/HCl pH 8 30 ml	NADP 5 mg	EC 1.1.1.49 G6PDH 0.02 ml	glucose-1-phosphate 80 mg	$MgCl_2$ 10 mg	MTT 10 mg PMS 2 mg agar 0.8 %
EC 3.5.4.4 ADA	1	7	4 h	0.05 M Tris/HCl pH 8 30 ml		EC 2.4.2.1 NP 0.02 ml EC 1.2.3.2 XO 0.02 ml	adenosine 20 ml		MTT 10 mg PMS 2 mg agar 0.8 %
EC 5.3.1.8 MPI	3	7	4 h	0.05 M Tris/HCl ph 8 30 ml	NADP 5 mg	EC 1.1.1.49 G6PDH 0.02 ml EC 5.3.1.9 GPI 0.02 ml	mannose-6-phosphate 25 mg	$MgCl_2$ 10 mg	MTT 10 mg PMS 2 mg agar 0.8 %
EC 5.3.1.9 GPI	3	7	4½ h	0.05 M Tris/HCl pH 8 30 ml	NADP 5 mg	EC 1.1.1.49 G6PDH 0.02 ml	fructose-6-phosphate 10 mg	$MgCl_2$ 10 mg	MTT 10 mg PMS 2 mg agar 0.8 %

Table 3. Allele frequencies found at 11 enzyme loci in populations of D. dorotocephala (SUF, PEN, BLA, SAL), D. jenkinsae (SFE), D. arizonensis (SAB), and D. tahitiensis (TAH) The number of individuals tested is indicated in brackets

Loci	*Alleles*	SUF (15)	PEN (37)	BLA (20)	SAL (25)	SFE (22)	SAB (18)	TAH (19)
Mdh-1	*97*	–	–	–	–	1.00	–	–
	100	1.00	1.00	1.00	1.00	–	1.00	–
	108	–	–	–	–	–	–	1.00
Mdh-2	*90*	–	–	–	–	1.00	–	–
	100	1.00	1.00	1.00	1.00	–	1.00	1.00
Idh-1	*94*	–	0.31	–	–	–	0.50	0.13
	100	1.00	0.69	1.00	1.00	1.00	0.50	0.87
Idh-2	*100*	1.00	1.00	1.00	0.80	1.00	–	–
	110	–	–	–	0.20	–	1.00	–
	135	–	–	–	–	–	–	1.00
G3pdh	*85*	–	–	–	–	–	1.00	–
	90	–	–	–	–	–	–	1.00
	93	–	–	–	–	1.00	–	–
	100	1.00	1.00	1.00	1.00	–	–	–
Got-1	*93*	–	–	–	–	1.00	1.00	–
	100	0.50	0.51	0.50	0.64	–	–	–
	110	0.50	0.49	0.50	0.36	–	–	–
	120	–	–	–	–	–	–	1.00
Ck	*95*	–	–	–	–	1.00	1.00	–
	100	1.00	1.00	0.50	0.90	–	–	–
	105	–	–	–	–	–	–	1.00
	120	–	–	0.50	0.10	–	–	–
Pgm-2	*1.00*	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ada	*43*	–	–	–	–	–	1.00	–
	48	–	–	–	–	1.00	–	–
	53	–	–	–	–	–	–	1.00
	100	1.00	1.00	1.00	1.00	–	–	–
Mpi	*85*	–	–	–	–	–	–	1.00
	97	–	–	–	–	1.00	1.00	–
	100	1.00	1.00	1.00	1.00	–	–	–
Gpi	*96*	–	–	–	–	–	–	1.00
	98	–	–	–	–	1.00	–	–
	100	1.00	1.00	1.00	1.00	–	–	–
	105	–	–	–	–	–	1.00	–
Ho		*0.09*	*0.11*	*0.18*	*0.09*	*0.00*	*0.09*	*0.02*

Table 4. Matrix of values of genetic distance according to Rogers (above the diagonal) and Nei (below the diagonal) between populations of D. dorotocephala s.s. (SUF, PEN, BLA, SAL), D. jenkinsae (SFE), D. arizonensis (SAB), and D. tahitiensis (TAH)

	SUF	PEN	BLA	SAL	SFE	SAB	TAH
SUF	–	0.028	0.045	0.040	0.715	0.670	0.726
PEN	0.009	–	0.074	0.069	0.744	0.641	0.732
BLA	0.024	0.035	–	0.068	0.703	0.657	0.714
SAL	0.007	0.017	0.022	–	0.730	0.648	0.716
SFE	1.276	1.365	1.252	1.323	–	0.591	0.830
SAB	1.099	1.078	1.074	1.021	0.871	–	0.761
TAH	1.309	1.373	1.284	1.286	1.759	1.448	–

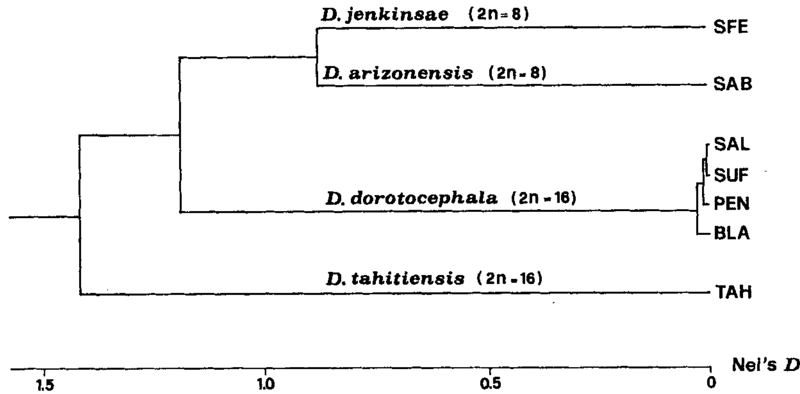

Fig. 1. UPGMA dendrogram from the values of NEI's D (Table 4), showing the genetic relationships among populations of four species of the subgenus *Girardia*: *Dugesia dorotocephala* s.s. (SAL, SUF, PEN, BLA), *D. jenkinsae* (SFE), *D. arizonensis* (SAB), and *D. tahitiensis* (TAH)

between any of the four species were found at the loci *Mdh-1, G3pdh, Ada,* and *Gpi*. The inter-taxa comparisons give values of NEI's genetic distance ranging from 0.871 to 1.759, as commonly found between congeneric, morphologically differentiated species.

D. arizonensis (namely the population from Sabino), considered strictly allied to *D. dorotocephala*, appears to be remarkably differentiated genetically from the latter (average NEI's $D = 1.068$), whereas it is more related to *D. jenkinsae* (NEI's $D = 0.871$), which has the same chromosome number (2n = 8).

NEI's genetic distances between the members of the *dorotocephala* group and *D. tahitiensis* range from 1.313 (for *D. dorotocephala* s.s.) to 1.759 (for *D. jenkinsae*), with an average of 1.507. The lowest value (1.313) is similar to that found between *D. dorotocephala* and *D. jenkinsae* ($D = 1.304$). These data indicate that the *D. dorotocephala* group includes species highly differentiated from each other. No clearcut distinction exists at the genetic level between this group and other species of the *Girardia* subgenus, such as *D. tahitiensis*.

The genetic relationships between the populations studied are given in Fig. 1, on the basis of UPGMA cluster analysis (SOKAL and SNEATH 1963), and in Fig. 2, on the basis of multidimensional scaling (GUTTMAN 1968).

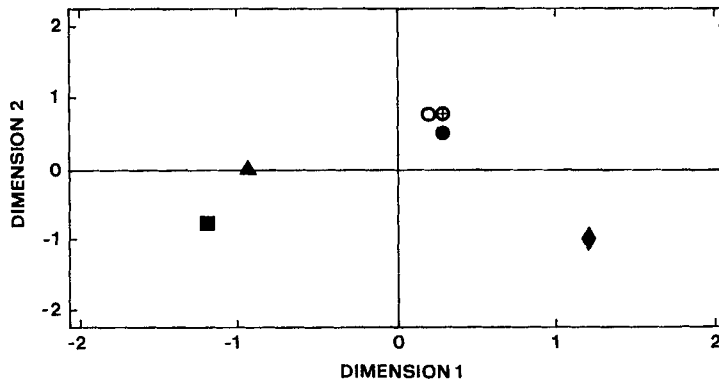

Fig. 2. Multidimensional scaling from the values of NEI's D (Table 4), showing the genetic relationships among populations of four species of the subgenus *Girardia*. *Dugesia dorotocephala* s.s.: SAL, SUF (open circle), PEN (crossed circle), BLA (solid circle); *D. arizonensis*: SAB (triangle); *D. jenkinsae*: SFE (square); *D. tahitiensis*: TAH (diamond)

Genetic data throw some light on the evolutionary relationships of the *Girardia* species considered. At the chromosome level, the so-called Robertsonian mechanisms cannot explain the evolution from the 2n = 16 karyotype to that with 2n = 8; this is because all chromosomes are two-armed in both karyotypes. Also, the hypothesis of a tetraploidization of *D. dorotocephala* from a diploid number 2n = 8 to 4n = 16 appears untenable. On the basis of allozyme data, the most likely hypothesis implies a common ancestor with 2n = 16. It would have given origin, by geographic isolation, to a North American line, *dorotocephala*-like, and to a Polynesian one, *tahitiensis*-like, both with 2n = 16. The former gave rise to species respectively with 2n = 16 (as *D. dorotocephala* s.s.) and, more recently, with 2n = 8 (as *D. arizonensis* and *D. jenkinsae*). The line with 2n = 8 seems to have originated in the southern part of the United States or perhaps in Mexico.

Further studies, including other species of the subgenus *Girardia*, both from America and the Pacific region, appear to be necessary to clarify the picture of the evolutionary relationships existing among these planarians.

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Zusammenfassung

Genetische Divergenz bei den Nordamerikanischen Planarien aus der Dugesia dorotocephala-Gruppe (Turbellaria, Tricladida, Paludicola)

Die genetische Differenzierung zwischen den Arten der *Dugesia (Girardia) dorotocephala*-Gruppe wurde mit Hilfe der Gelelektrophorese untersucht und mit der zu einer anderen Planarien-Art, *D. tahitiensis*, die ebenfalls zur Untergattung *Girardia* gehört, verglichen. Die untersuchten Arten waren: *D. dorotocephala s.s.* (2n = 16), *D. arizonensis* (2n = 8), *D. jenkinsae* (2n = 8) und die oben erwähnte *D. tahitiensis* (2n = 16). Die ersten drei Arten sind in Nordamerika verbreitet. Bei ihnen treten mit unterschiedlichen Anteilen fissipare und geschlechtliche Individuen auf. *D. tahitiensis* bewohnt Polynesien und vermehrt sich ausschließlich asexuell. Insgesamt wurden folgende 11 Enzymloci analysiert: *Mdh-1, Mdh-2, Idh-1, Idh-2, G3pdh, Got-1, Ck, Pgm-2, Ada, Mpi* und *Gpi*. Insgesamt erwies sich der durchschnittliche Heterozygotiegrad in den untersuchten Populationen als sehr niedrig (Ho = 0 bis 0.18; Durchschnitt 0.08). In den sexuellen Populationen (außer in denen von Tahiti) wurde bei allen Individuen eine fixierte Heterozygotie an ein bis zwei Loci gefunden. Die genetische Divergenz zwischen den untersuchten Arten ist aber sehr hoch und viele Loci sind durch diskriminierende Allele vertreten (*D* nach NEI zwischen 0.871 bis 1.759). Die Populationen von *D. dorotocephala* s.s. sind hingegen untereinander genetisch relativ homogen (*D* = 0.019) und es zeigt sich kein Zusammenhang zwischen der Größe von *D* und der geographischen Entfernung oder der Reproduktionsweise. Die genetische Distanz zwischen *D. tahitiensis*, einer Art, die nicht zur *D. dorotocephala*-Gruppe gehört, und *D. dorotocephala* s.s. ist 1.314; dies ist ein Wert, der dem Distanzwert von *D* = 1.303 zwischen den zwei Arten der *D. dorotocephala*-Gruppe, *D. dorotocephala* s.s. und *D. jenkinsae* sehr ähnlich ist. Die genetische Verwandtschaft zwischen den Populationen wurde durch eine UPGMA-Cluster-Analyse und durch das Multi-dimensionale-Scaling-Verfahren ermittelt und in einem Dendrogramm dargestellt. Es erscheint wahrscheinlich, daß die nordamerikanischen Arten mit 2n = 8 auf einen *dorotocephala*-ähnlichen Vorfahren mit einem Chromosomensatz 2n = 16 zurückgehen. Es wird auch angenommen, daß dieser Vorfahre und eine *tahitiensis*-ähnliche Form (mit 2n = 16) dann wieder aus einem früheren gemeinsamen Vorfahren durch geographische Isolation entstanden sind.

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