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PLOS ONE

A first AFLP-Based Genetic Linkage Map for Brine Shrimp *Artemia franciscana* and Its Application in Mapping the Sex Locus

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Abstract

We report on the construction of sex-specific linkage maps, the identification of sex-linked markers and the genome size estimation for the brine shrimp *Artemia franciscana*. Overall, from the analysis of 433 AFLP markers segregating in a 112 full-sib family we identified 21 male and 22 female linkage groups ($2n = 42$), covering 1,041 and 1,313 cM respectively. Fifteen putatively homologous linkage groups, including the sex linkage groups, were identified between the female and male linkage map. Eight sex-linked AFLP marker alleles were inherited from the female parent, supporting the hypothesis of a WZ–ZZ sex-determining system. The haploid *Artemia* genome size was estimated to 0.93 Gb by flow cytometry. The produced *Artemia* linkage maps provide the basis for further fine mapping and exploring of the sex-determining region and are a possible marker resource for mapping genomic loci underlying phenotypic differences among *Artemia* species.

Citation: De Vos S, Bossier P, Van Stappen G, Vercauteren I, Sorgeloos P, et al. (2013) A first AFLP-Based Genetic Linkage Map for Brine Shrimp *Artemia franciscana* and Its Application in Mapping the Sex Locus. PLoS ONE 8(3): e57585. doi:10.1371/journal.pone.0057585

Editor: Zhanjiang Liu, Auburn University, United States of America

Received October 25, 2012; **Accepted** January 22, 2013; **Published** March 4, 2013

Funding: The authors acknowledge the support of Ghent University for the Bijzonder Onderzoeksfonds (BOF) of Stephanie De Vos. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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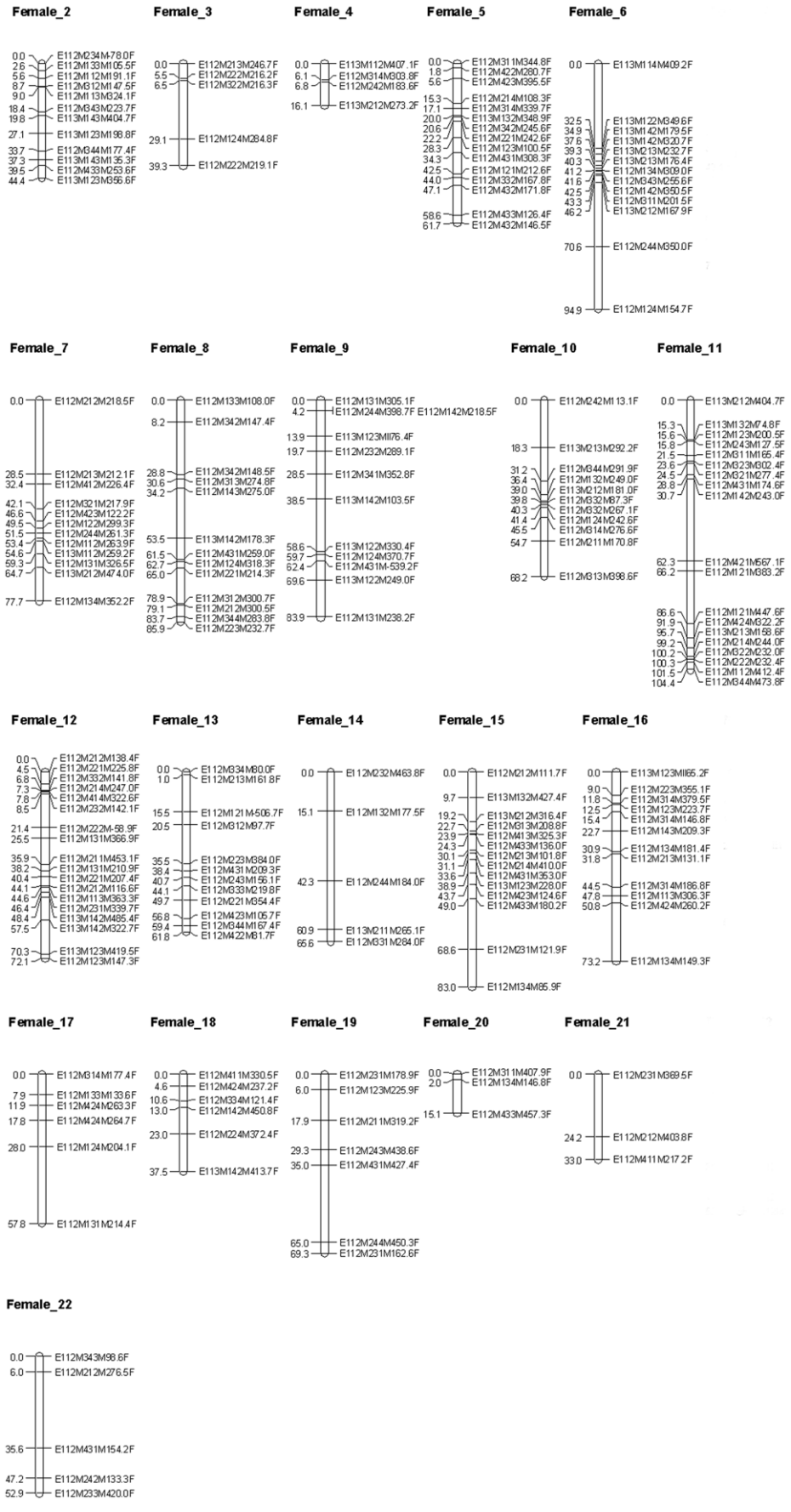
Introduction

Artemia, known as brine shrimp, is a genus of small planktonic crustaceans found worldwide in natural salt lakes and salterns [1]. Their larvae (nauplii) are the most commonly used live food in aquaculture activities, specifically for larval growth of more than 85% of the marine species reared in aquaculture [2,3]. Adult *Artemia* survive extreme salinities, while their encysted embryos (cysts), produced under stressful conditions, have a unique tolerance for high doses of UV and ionizing radiation, anoxia, thermal extremes and desiccation-hydration cycles [4–6]. Cysts remain viable for years and produce nauplii within 24 h after hydration.

An overview of *Artemia* cytogenetics, DNA content and available molecular tools is provided. Six different sexually dimorphic species can be found in the *Artemia* genus, among which *Artemia franciscana Kellogg*, 1906 [4] and several obligate parthenogenetic *Artemia* populations ranging in ploidy from 2n to 5n [7]. All sexually dimorphic *Artemia* species are diploids with $2n = 42$, except *A. persimilis* ($2n = 44$) [8]. The *Artemia* genome size has been assessed with two different techniques producing discordant estimates: 2.93 Gb (3 pg) by Feulgen densitometry [9] and 1.47 Gb (1.5 pg) by DNA reassociation kinetics [10]. Despite the use of flow cytometry in the most recent evaluations of crustacean genome size [11–13], so far no flow cytometry-based estimates of the *Artemia* genome have been published. To date, genomic resources for *Artemia* have been limited to RAPD [14,15], RFLP [16], AFLP [17,18], microsatellite markers [19] and the 15,822 bp mitochondrial genome sequence [20,21].

In crustaceans, three major genetic sex determination systems have been suggested by cytogenetics and sex-linked markers: WZ-ZZ (females are the heterogametic sex), XX-XY (males are the heterogametic sex) and androdioecy (a mix of ZZ males and WZ hermaphrodites, as in *Eulimnadia texana*) [22]. Examples of crustaceans with an XX-XY sex-determining system are decapods such as the Chinese mitten crab *Eriocheir sinensis* [23,24], terrestrial isopods and the amphipods *Orchestia cavimana* and *O. gammarellus* [25]. However, a WZ-ZZ sex-determining system has been found in decapods such as *Litopenaeus vannamei* [26], tiger shrimp *Penaeus monodon* [27], *Macrobrachium rosenbergii* [28], kuruma prawn *Penaeus japonicus* [29], Australian red claw crayfish *Cherax quadricarinatus* [24] and in isopods like *Armadillidium vulgare* and all Valvifera, except *Saduria entomon* [30]. In bisexual *Artemia*, female heterogamety has been suggested previously by observation of sexual heterochromosomes in *A. salina* [31], *A. franciscana* and *A. persimilis* [8]; by crossing experiments with *A. franciscana* showing a recessive sex-linked trait called "white eye" [32] and by karyotyping and heterochromatin experiments showing one heterochromatic block in female and two in male *A. persimilis* [8].

Over the last decade, linkage maps have been developed for a number of crustaceans such as *Daphnia pulex* [33], *D. magna* [34], *Tigriopus californicus* [35], *P. monodon* [36–38], *L. vannamei* [39–41], *Fenneropenaeus chinensis* [42,43] and *P. japonicus* [44]. Sex-linked markers have been found in males of the isopod *Mysis relicta* [45] and of *Triops cancriformis* [46]. In female crustaceans, sex-linked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8.7 E112M312M147.5F
9.0 E112M113M324.1F
18.4 E112M343M223.7F
19.8 E113M143M404.7F
27.1 E113M123M198.8F
33.7 E112M344M177.4F
37.3 E113M143M135.3F
39.5 E112M433M253.6F
44.4 E113M123M356.6F
Female_3
0.0 E112M213M246.7F
5.5 E112M222M216.2F
6.5 E112M322M216.3F
29.1 E112M124M284.8F
39.3 E112M222M219.1F
Female_4
0.0 E113M112M407.1F
6.1 E112M314M303.8F
6.8 E112M242M183.6F
16.1 E113M212M273.2F
Female_5
0.0 E112M311M344.8F
1.8 E112M422M280.7F
5.6 E112M423M395.5F
15.3 E112M214M108.3F
17.1 E112M314M339.7F
20.0 E113M132M348.9F
20.6 E112M342M245.6F
22.2 E112M221M242.6F
28.3 E112M123M100.5F
34.3 E112M431M308.3F
42.5 E112M121M212.6F
44.0 E112M332M167.8F
47.1 E112M432M171.8F
58.6 E112M433M126.4F
61.7 E112M432M146.5F
Female_6
0.0 E113M114M409.2F
32.5 E113M122M349.6F
34.9 E113M142M179.5F
37.6 E113M142M320.7F
39.3 E113M213M232.7F
40.3 E113M213M176.4F
41.2 E112M134M309.0F
41.6 E112M343M255.6F
42.5 E112M142M350.5F
43.3 E112M311M201.5F
46.2 E113M212M167.9F
70.6 E112M244M350.0F
94.9 E112M124M154.7F
Female_7
0.0 E112M212M218.5F
28.5 E112M213M212.1F
32.4 E112M412M226.4F
42.1 E112M321M217.9F
46.6 E112M423M122.2F
49.5 E112M122M299.3F
51.5 E112M244M261.3F
53.4 E112M112M263.9F
54.6 E113M112M259.2F
59.3 E112M131M326.5F
64.7 E113M212M474.0F
77.7 E112M134M352.2F
Female_8
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8.2 E112M342M147.4F
28.8 E112M342M148.5F
30.6 E112M313M274.8F
34.2 E112M143M275.0F
53.5 E113M142M178.3F
61.5 E112M431M259.0F
62.7 E112M124M318.3F
65.0 E112M221M214.3F
78.9 E112M312M300.7F
79.1 E112M212M300.5F
83.7 E112M344M283.8F
85.9 E112M223M232.7F
Female_9
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4.2 E112M244M398.7F E112M142M218.5F
13.9 E113M123MII76.4F
19.7 E112M232M289.1F
28.5 E112M341M352.8F
38.5 E113M142M103.5F
58.6 E113M122M330.4F
59.7 E112M124M370.7F
62.4 E112M431M-539.2F
69.6 E113M122M249.0F
83.9 E112M131M238.2F
Female_10
0.0 E112M242M113.1F
18.3 E113M213M292.2F
31.2 E112M344M291.9F
36.4 E112M132M249.0F
39.0 E113M212M181.0F
39.8 E112M332M87.3F
40.3 E112M332M267.1F
41.4 E112M124M242.6F
45.5 E112M314M276.6F
54.7 E112M211M170.8F
68.2 E112M313M398.6F
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0.0 E113M212M404.7F
15.3 E113M132M74.8F
15.6 E112M123M000.5F
15.8 E112M243M127.5F
21.5 E112M311M165.4F
23.6 E112M323M302.4F
24.5 E112M321M277.4F
28.8 E112M431M174.6F
30.7 E112M142M243.0F
62.3 E112M421M567.1F
66.2 E112M121M383.2F
86.6 E112M121M447.6F
91.9 E112M424M322.2F
95.7 E113M213M158.6F
99.2 E112M214M244.0F
100.2 E112M322M232.0F
100.3 E112M222M232.4F
101.5 E112M112M412.4F
104.4 E112M344M473.8F
Female_12
0.0 E112M212M138.4F
4.5 E112M221M225.8F
6.8 E112M332M141.8F
7.3 E112M214M247.0F
7.8 E112M414M322.6F
8.5 E112M232M142.1F
21.4 E112M222M-58.9F
25.5 E112M131M366.9F
35.9 E112M211M453.1F
38.2 E112M131M210.9F
40.4 E112M221M207.4F
44.1 E112M212M116.6F
44.6 E112M113M363.3F
46.4 E112M231M339.7F
48.4 E113M142M485.4F
57.5 E113M142M322.7F
70.3 E113M123M419.5F
72.1 E112M123M147.3F
Female_13
0.0 E112M334M80.0F
1.0 E112M213M161.8F
15.5 E112M121M-506.7F
20.5 E112M312M97.7F
35.5 E112M223M384.0F
38.4 E112M431M209.3F
40.7 E112M243M156.1F
44.1 E112M333M219.8F
49.7 E112M221M354.4F
56.8 E112M423M105.7F
59.4 E112M344M167.4F
61.8 E112M422M81.7F
Female_14
0.0 E112M232M463.8F
15.1 E112M132M177.5F
42.3 E112M244M184.0F
60.9 E113M211M265.1F
65.6 E112M331M284.0F
Female_15
0.0 E112M212M111.7F
9.7 E113M132M427.4F
19.2 E113M212M316.4F
22.7 E112M313M208.8F
23.9 E112M413M325.3F
24.3 E112M433M136.0F
30.1 E112M213M101.8F
31.1 E112M214M410.0F
33.6 E112M431M353.0F
38.8 E113M123M228.0F
43.7 E112M423M124.6F
49.0 E112M433M180.2F
68.6 E112M231M121.9F
83.0 E112M134M85.9F
Female_16
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9.0 E112M223M355.1F
11.8 E112M314M379.5F
12.5 E112M123M223.7F
15.4 E112M314M146.8F
22.7 E112M143M209.3F
30.9 E112M134M181.4F
31.8 E112M213M131.1F
44.5 E112M314M186.8F
47.8 E112M113M306.3F
50.8 E112M424M260.2F
73.2 E112M134M149.3F
Female_17
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11.9 E112M424M263.3F
17.8 E112M424M264.7F
28.0 E112M124M204.1F
57.8 E112M131M214.4F
Female_18
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4.6 E112M424M237.2F
10.6 E112M334M121.4F
13.0 E112M142M450.8F
23.0 E112M224M372.4F
37.5 E113M142M413.7F
Female_19
0.0 E112M231M178.9F
6.0 E112M123M225.9F
17.9 E112M211M319.2F
29.3 E112M243M438.6F
35.0 E112M431M427.4F
65.0 E112M244M450.3F
69.3 E112M231M162.6F
Female_20
0.0 E112M311M407.9F
2.0 E112M134M146.8F
15.1 E112M433M457.3F
Female_21
0.0 E112M231M369.5F
24.2 E112M212M403.8F
33.0 E112M411M217.2F
Female_22
0.0 E112M343M98.6F
6.0 E112M212M276.5F
35.6 E112M431M154.2F
47.2 E112M242M133.3F
52.9 E112M233M420.0F

Figure 1. *Artemia franciscana* autosomal female linkage groups Twenty-one linkage groups representing the *Artemia franciscana* autosomal genome containing markers originating from female parental strain Vinh Chau (ARC1349). Each AFLP marker is represented by (1) a code referring to the corresponding PC (Table S1), followed by (2) the molecular size of the fragment in nucleotides and (3) the type of parental marker (female marker, tagged as "F"). Cumulative marker distances (cM) are indicated on the left.
doi:10.1371/journal.pone.0057585.g001

markers have been found in the isopods *Paracerceis sculpta* [47] and *Jaera ischiosetosa* [48], in the crab *Cancer setosus* [49], in penaeid shrimps *L. vannamei* [41] and *P. monodon* [27] and in giant freshwater prawn *M. rosenbergii* [50]. Moreover, a hermaphrodite-determining allele has been studied in the androdioecious branchiopod *E. texana* [51]. So far, neither linkage maps, nor trait-linked markers including sex-linked markers have been identified in *Artemia* [8].

Genetic linkage maps are invaluable in forward genetic analyses for the identification of the genomic loci responsible for phenotypic differences. From this perspective, *Artemia* offers a number of major advantages for time-effective generating of experimental mapping populations and for mapping natural allelic variation. They have a short generation time (2–4 weeks), offspring production of several hundred individuals per brood, storability of cysts for years, easy breeding in large numbers and levels of genetic variability that are among the highest within crustaceans [16,41,52]. In addition, we expect that forward genetic approaches in *Artemia* are not only restricted to *Artemia*-specific traits, but are also valuable for mapping traits such as sex, *Vibrio* pathogen resistance and growth rate, segregating in commercially important crustaceans. We believe therefore, that *Artemi*a could be a useful model species for other crustaceans.

In the present study, we report on a first AFLP-based linkage map of *A. franciscana*. We additionally present eight sex-linked markers that disclose the linkage group corresponding to the W chromosome and confirm female heterogamety in *A. franciscana*. Finally, we report on the estimation of the *A. franciscana* genome size by flow cytometry.

Materials and Methods

Mapping population

Cyst material of the *A. franciscana* strains from San Francisco Bay, USA (SFB; ARC1364) and Vinh Chau, Vietnam (VC; ARC1349) was obtained from the Laboratory of Aquaculture & Artemia Reference Center cyst bank (http://www.aquaculture.ugent.be). The SFB strain was first introduced in Vinh Chau, Vietnam in 1982, eventually resulting in the new VC strain in the late 1980̀s [53]. First, cysts from both strains were hatched separately in aerated seawater (28°C, salinity 35 $g.l^{-1}$). The instar I nauplii of each strain were then harvested and reared for a week in aerated seawater with added sea salt (Instant Ocean®, 28°C, final salinity 70 $g.l^{-1}$) and fed with *Tetraselmis suecica*, a marine unicellular green alga. The *Artemia* were subsequently transferred to individual Falcon tubes and kept there under the same conditions for seven days until sexual maturation. A controlled cross between VC (♀) and SFB (♂) was then made, resulting in F_1 progeny that was collected over a sieve every two days and grown until maturity under the same conditions as the parental generation. Adult F_1 progeny was rinsed with sterile distilled water and the phenotypic sex of each F_1 offspring individual was determined visually. For gut evacuation before DNA extraction, the offspring and parents were starved during 24 h, followed by removal of the brood pouch in females. Parents and progeny were stored individually at −20°C.

DNA extraction

DNA was extracted from parents and their 112 F_1 offspring according to a modified CTAB-method for shrimp tissue [54]. Briefly: to each sample, ground in liquid N_2, 150 µl of CTAB buffer was added. After homogenization, 750 µl of extra CTAB buffer was added and the mix was left at 25°C for 30 min. PCA solution was added (600 µl; 25:24:1 phenol/chloroform/isoamylalcohol). After centrifugation, 800 µl of the upper aqueous phase was added to 600 µl of CA solution (24:1 chloroform/isoamylalcohol) and the mix was homogenized. To 700 µl of the upper aqueous phase, 630 µl of isopropanol was added. The mix was incubated for 1 h at −70°C. After centrifugation, the pellet was washed with 600 µl of ethanol 70%, air-dried in a 60°C oven and resuspended in 20 µl of sterile distilled water. DNA quality and concentration were assessed on a 1% agarose gel.

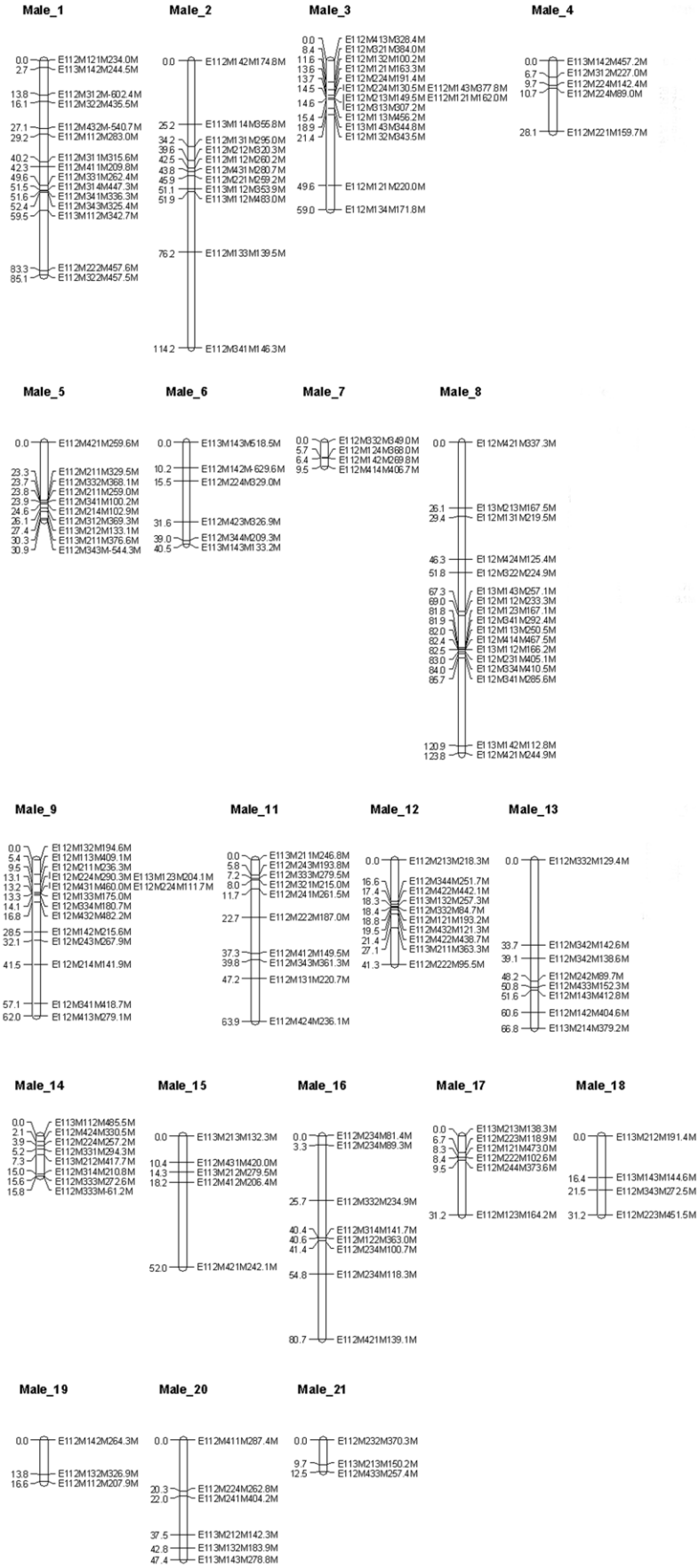
Segregation analysis and linkage mapping

AFLP analysis with fluorescent dye detection was performed on a LI-COR long read-IR^2 4200 (LI-COR Biosciences) as described by Vuylsteke et al. [55]. Sixty-five *Eco*RI+3/*Mse*I+3 primer combinations (PCs) listed in Table S1 were used. AFLP analysis of parents and 112 offspring was done on two separate 64-lane gels per PC.

The degree of polymorphism between the two parental strains was estimated based on AFLP fragments amplified by four PCs (E112M212, E112M213, E112M233 and E112M234).

AFLP markers were scored using the specific image analysis software AFLP-Quantar*Pro* (http://www.keygene-products.com) as described in Vuylsteke et al. [55]. Each AFLP marker was identified by (1) a code referring to the corresponding PC (Table S1), followed by (2) the molecular size of the fragment in nucleotides as estimated by AFLP-Quantar*Pro*, and (3) a tag referring to the type of marker. Parental AFLP markers segregating 1:1 in the F_1 progeny are heterozygous in either the female (female marker, tagged as "F") or the male parent (male marker, tagged as "M") and homozygous absent in the other parent. AFLP markers heterozygous in one of the parents and homozygous present in the other were not included in the linkage analysis, because heterozygotes could not be reliably discriminated from individuals homozygous for the "band present" allele. No tag was used for biparental markers, which are heterozygous in both parents and thus, segregate 1:2:1 in the F_1 progeny. Parental and biparental AFLP markers were scored co-dominantly based on relative fragment intensities resulting in more genetic information compared to dominant (present/absent) scoring and hence, speeding up the mapping process [55]. However, biparental markers were scored dominantly when the heterozygotes could not reliably be discriminated from the individuals homozygous for the "band present" allele.

Linkage and segregation analyses were performed using the software package Joinmap 4 [56]. The mapping population type was set to CP (i.e. a population resulting from a cross between two heterogeneously heterozygous and homozygous diploid parents, linkage phases originally unknown). The segregation type was encoded according to Joinmap 4 recommendations [56]. A logarithm of the odds (LOD) threshold range between 2.0 and 14.0 was initially used to group parental markers. Only linkage groups containing at least three markers were considered for map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13.8 E112M312M-602.4M
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29.2 E112M112M283.0M
40.2 E112M311M315.6M
42.3 E112M411M209.6M
49.6 E112M331M262.4M
51.5 E112M314M447.3M
51.6 E112M341M336.3M
52.4 E112M343M325.4M
59.5 E113M112M342.7M
83.3 E112M222M457.6M
85.1 E112M322M457.5M
Male_2
0.0 E112M142M174.8M
25.2 E113M114M355.8M
34.2 E112M131M295.0M
39.6 E112M212M320.3M
42.5 E112M112M260.2M
43.8 E112M431M280.7M
45.9 E112M221M259.2M
51.1 E113M112M353.9M
51.9 E113M112M483.0M
76.2 E112M133M139.5M
114.2 E112M341M146.3M
Male_3
0.0 E112M413M328.4M
8.4 E112M321M384.0M
11.6 E112M132M100.2M
13.6 E112M121M163.3M
13.7 E112M224M191.4M
14.5 E112M224M130.5M E112M143M377.8M
14.6 E112M213M149.5M E112M121M162.0M
15.4 E112M313M907.2M
18.9 E112M113M456.2M
21.4 E113M143M344.8M
49.6 E112M132M343.5M
59.0 E112M121M220.0M
59.0 E112M134M171.8M
Male_4
0.0 E113M142M457.2M
6.7 E112M312M227.0M
9.7 E112M224M142.4M
10.7 E112M224M89.0M
28.1 E112M221M159.7M
Male_5
0.0 E112M421M259.6M
23.3 E112M211M329.5M
23.7 E112M332M368.1M
23.8 E112M211M259.0M
23.9 E112M341M100.2M
24.6 E112M214M102.9M
26.1 E112M312M369.3M
27.4 E113M212M133.1M
30.3 E113M211M376.6M
30.9 E112M343M-544.3M
Male_6
0.0 E113M143M518.5M
10.2 E112M142M-629.6M
15.5 E112M224M329.0M
31.6 E112M423M326.9M
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40.5 E113M143M133.2M
Male_7
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Male_8
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51.8 E112M322M224.9M
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81.9 E112M341M292.4M
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82.4 E112M414M467.5M
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84.0 E112M334M410.5M
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123.8 E112M421M244.9M
Male_9
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5.4 E112M113M409.1M
9.5 E112M211M236.3M
13.1 E112M224M290.3M E113M123M204.1M
13.2 E112M431M460.0M E112M224M111.7M
13.3 E112M133M175.0M
14.1 E112M334M180.7M
16.8 E112M432M482.2M
28.5 E112M142M215.6M
32.1 E112M243M267.9M
41.5 E112M214M141.9M
57.1 E112M341M418.7M
62.0 E112M413M279.1M
Male_11
0.0 E113M211M246.8M
5.8 E112M243M193.8M
7.2 E112M333M279.5M
8.0 E112M321M215.0M
11.7 E112M241M261.5M
22.7 E112M222M187.0M
37.3 E112M412M149.5M
39.8 E112M343M361.3M
47.2 E112M131M220.7M
63.9 E112M424M236.1M
Male_12
0.0 E112M213M218.3M
16.6 E112M344M251.7M
17.4 E112M422M442.1M
18.3 E113M132M257.3M
18.4 E112M332M64.7M
18.8 E112M121M193.2M
19.5 E112M432M121.3M
21.4 E112M422M438.7M
27.1 E113M211M363.3M
41.3 E112M222M95.5M
Male_13
0.0 E112M332M129.4M
33.7 E112M342M142.6M
39.1 E112M342M138.6M
48.2 E112M242M89.7M
50.8 E112M433M152.3M
51.6 E112M143M412.8M
60.6 E112M142M404.6M
66.8 E113M214M379.2M
Male_14
0.0 E113M112M485.5M
2.1 E112M424M330.5M
3.9 E112M224M257.2M
5.2 E112M331M294.3M
7.3 E113M212M417.7M
15.0 E112M314M210.8M
15.6 E112M333M272.6M
15.8 E112M333M-61.2M
Male_15
0.0 E113M213M132.3M
10.4 E112M431M420.0M
14.3 E113M212M279.5M
18.2 E112M412M206.4M
52.0 E112M421M242.1M
Male_16
0.0 E112M234M81.4M
3.3 E112M234M89.3M
25.7 E112M332M234.9M
40.4 E112M314M141.7M
40.6 E112M122M363.0M
41.4 E112M234M100.7M
54.8 E112M234M118.3M
80.7 E112M421M139.1M
Male_17
0.0 E113M213M138.3M
6.7 E112M223M118.9M
8.3 E112M121M473.0M
8.4 E112M222M102.6M
9.5 E112M244M373.6M
31.2 E112M123M164.2M
Male_18
0.0 E113M212M191.4M
16.4 E113M143M144.6M
21.5 E112M343M272.5M
31.2 E112M223M451.5M
Male_19
0.0 E112M142M264.3M
13.8 E112M132M326.9M
16.6 E112M112M207.9M
Male_20
0.0 E112M411M287.4M
20.3 E112M224M262.8M
22.0 E112M241M404.2M
37.5 E113M212M142.3M
42.8 E113M132M183.9M
47.4 E113M143M278.8M
Male_21
0.0 E112M232M370.3M
9.7 E113M213M150.2M
12.5 E112M433M257.4M

Figure 2. *Artemia franciscana* autosomal male linkage groups. Twenty linkage groups representing the *Artemia franciscana* autosomal genome containing markers originating from male parental strain San Francisco Bay (ARC1364). Each AFLP marker is represented by (1) a code referring to the corresponding PC (Table S1), followed by (2) the molecular size of the fragment in nucleotides and (3) the type of parental marker (male marker, tagged as "M"). Cumulative marker distances are indicated on the left (cM).
doi:10.1371/journal.pone.0057585.g002

construction. Segregation distortion of markers was tested by using a χ^2-test as implemented in Joinmap 4. Graphical presentation of linkage groups was done with the software MapChart [57].

Artemia genome size estimation by flow cytometry

The haploid genome size (GS) of *Artemia* was assessed against the rainbow trout (haploid GS 2.4–3.0 pg or 2.35−2.93 Gb [58]) and the chicken genome (haploid GS 1.07 pg or 1.05 Gb [59]), both used as internal standards.

The consistency of the used method was assessed by calibrating rainbow trout nuclei (2 µl of freshly drawn heparinized *Oncorynchus mykiss* blood) against chicken erythrocyte nuclei (2 µl of 10x diluted BioSure®CEN singlet, *Gallus gallus domesticus*, Rhode Island Red female).

Each of the four *Artemia* individuals (i.e. four full-sib males from the VC (♀) x SFB (♂) cross) were chopped together with internal standard material using a razor in 1 ml of Galbraiths buffer as described in Dolezel and Bartos [60]. Cell suspensions were filtered through a 30 µm mesh, put on ice and nuclei were co-stained in the dark for 2 min with 50 µl of fluorescent DNA stain Propidium Iodide (Sigma-Aldrich PI solution in water 1 mg/ml). The use of PI staining on *A. franciscana* (GC% 32) [61], *O. mykiss* (GC% 42) [58] and *G. domesticus* (GC% 47) [62] was chosen to avoid a GC content-linked bias, as occurs with DAPI staining [60]. At least 5,000 nuclei were analyzed for each co-stained sample, using a Modular Flow cytometer and cell sorter (MoFlo Legacy, Cytomation) with a 488 nm Argon laser and PI emission bandpass filter of 580/30 nm. Instrument calibration was performed using Flow-check Fluorospheres (Beckman Coulter) and internal standards. Fluorescence of the nuclei was recorded linearly with the software Summit v4.3. For each co-stained sample, fluorescence histograms were generated and mean fluorescence values were calculated with the flow cytometry data analysis software Cyflogic 1.2.1. The haploid GS of for each *Artemia* sample was calculated according to the following formula [13]: $GS = \frac{F_S \times F_{is}}{F_{is}}$, where F_s is the mean fluorescence of the sample and F_{is} is the mean fluorescence of the internal standard.

Results

Segregation analysis and linkage mapping

A total of 65 AFLP PCs resulted in a total of 531 markers, of which 433 were parental (239 female, 194 male) and 98 markers were biparental. Based on only four primer combinations (PCs) yielding 180 AFLP fragments, 36% of the fragments segregated between both parents.

First, a parental map including only parental markers was constructed. Summary statistics for the parental maps are listed in Table 1. The grouping of parental markers at a LOD score ranging from 5.0 to 6.0 resulted in a number of linkage groups corresponding with the haploid chromosome number (n = 21). The female map, containing 225 markers (Figure 1), resulted in 22 "female" linkage groups (LG) spanning 1,312.9 cM; the male map, containing 181 markers (Figure 2), resulted in 21 "male" LG spanning 1,041.3 cM. Twenty-eight percent of the analyzed parental markers showed significant ($p<0.05$; χ^2 test) segregation distortion. Male markers were more often distorted than female markers (31% resp. 25%). Some larger genomic regions did not contain any markers (e.g. 32.5 cM in LG Female_6, Figure 1; 38.0 cM in LG Male_2, Figure 2), despite the low median inter-marker distances of 3.9 and 3.1 cM for the female and the male linkage map (Table 1).

Next, an integrated map was created including the 98 biparental markers and 406 previously mapped parental markers (Figure 3). By including biparental markers, groups consistent with

Table 1. Statistics for the female and male linkage maps.

		Female (Vinh Chau)	Male (San Francisco Bay)
No. of linkage groups		22	21
No. of markers mapped per linkage group	Min	3	3
	Max	19	17
	Median	12	8
	Mean	10	9
	Total	225	181
Size of linkage groups (cM)	Min	15.1	9.5
	Max	104.4	123.8
	Median	63.7	44.4
	Mean	59.7	52.1
	Total	1312.9	1041.3
Intermarker distance (cM)	Min	0.0	0.0
	Max	32.5	38.0
	Median	3.9	3.1
	Mean	6.5	6.6

doi:10.1371/journal.pone.0057585.t001

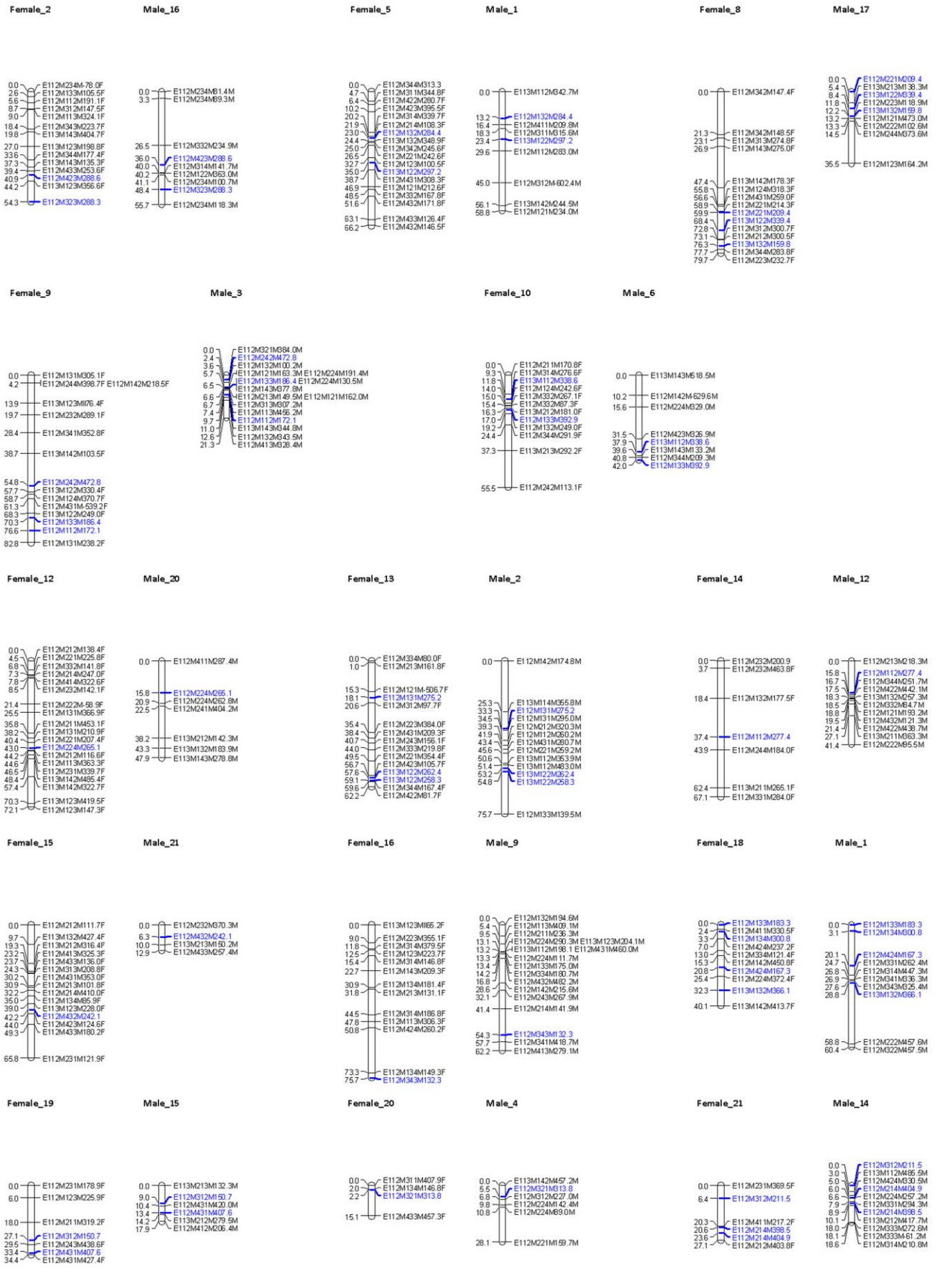
Female_2
Male_16
Female_5
Male_1
Female_8
Male_17
Female_9
Male_3
Female_10
Male_6
Female_12
Male_20
Female_13
Male_2
Female_14
Male_12
Female_15
Male_21
Female_16
Male_9
Female_18
Male_1
Female_19
Male_15
Female_20
Male_4
Female_21
Male_14

Figure 3. *Artemia franciscana* homologous autosomal male and female linkage groups. Fourteen homologous autosomal linkage group pairs. Each AFLP marker was identified by (1) a code referring to the corresponding PC (Table S1), followed by (2) the molecular size of the fragment in nucleotides and (3) the type of marker (female marker, tagged as "F", male marker, tagged as "M", biparental marker, no tag). Common biparental markers are indicated in blue. Cumulative marker distances are indicated on the left (cM).
doi:10.1371/journal.pone.0057585.g003

linkage groups of the parental map were obtained at a LOD threshold ranging between 6 and 10. Sixty-nine percent of the biparental marker loci showed significant ($p<0.05$; χ^2 test) segregation distortion. These loci were still included in map construction and evaluated for quality afterwards, since significant segregation distortion is inherent to relatively small experimental mapping population sizes of ~100 individuals. Forty-nine biparental markers (50%) could be mapped in the female as well as in the male map, identifying 15 homologous linkage groups including the sex linkage groups (Figure 3, Figure 4).

Mapping of the sex locus

Staelens et al. [27] described segregation patterns of sex-linked AFLP markers that unequivocally differentiate the WZ-ZZ and XX-XY sex-determination system. We observed eight AFLP markers, spanning a region of 0.2 cM on LG Female_1 (markers in green, Figure 4) segregating according to pattern 1 and a single marker (E112M122M167.3F) according to pattern 2. Both segregation patterns are expected under the assumption of female heterogamety. None of the markers segregated according to patterns 6, 7 and 8, expected under the assumption of male heterogamety. The male linkage group Male_10 was identified as homologous to Female_1 (Figure 4). In conclusion, the observed segregation patterns of sex-linked AFLP markers strongly favour female over male heterogamety in *Artemia*.

A. franciscana genome size estimation by flow cytometry

Using trout blood as the internal standard, the haploid female chicken genome size (GS) determined by flow cytometry was 1.05 Gb (1.07 pg) as previously reported for female chicken [59]. We preferred rainbow trout nuclei as the internal standard in the assessment of the *Artemia* GS because their fluorescence values did not overlap with those of *Artemia*, as was the case with fluorescence values obtained from chicken nuclei. Using rainbow trout nuclei as the internal standard, the *A. franciscana* haploid genome size was estimated to 0.93±0.09 Gb (0.97±0.09 pg; n = 4). Fluorescence histograms for each sample and for chicken are shown in Figure 5. Fluorescence peaks were relatively broad due to cell debris from the previously frozen *Artemia* individuals, but average DNA content estimates were consistent throughout the different samples, shown by the small standard error.

Discussion

We present the first sex-specific AFLP linkage maps and sex-linked markers as well as a consistent genome size (GS) estimation for the brine shrimp *A. franciscana*.

The linkage analysis of 433 parental AFLP markers segregating in a 112 full-sib family identified 21 male and 22 female linkage groups, corresponding very well with the haploid chromosome number in *A. franciscana* (2n = 42) [8]. Most likely, the markers in small linkage groups (LG) such as Female_20 (Figure 1) would join one of the other 21 LG by adding more markers to the female map. More female than male markers were generated, suggesting that maternal *A. franciscana* strain Vinh Chau (VC) has more unique alleles compared to the paternal strain San Francisco Bay (SFB). This seems a logical consequence of the SFB origins of VC. The level of polymorphism between the two *A. franciscana* parental strains was estimated at 36%, which is in the range of 9–50% estimated previously by Kappas et al. [53]. Given their high marker density, the produced genetic maps are adequate for the anchoring of *Artemia* genome sequences to facilitate the future construction of physical maps for each of the 21 chromosomes.

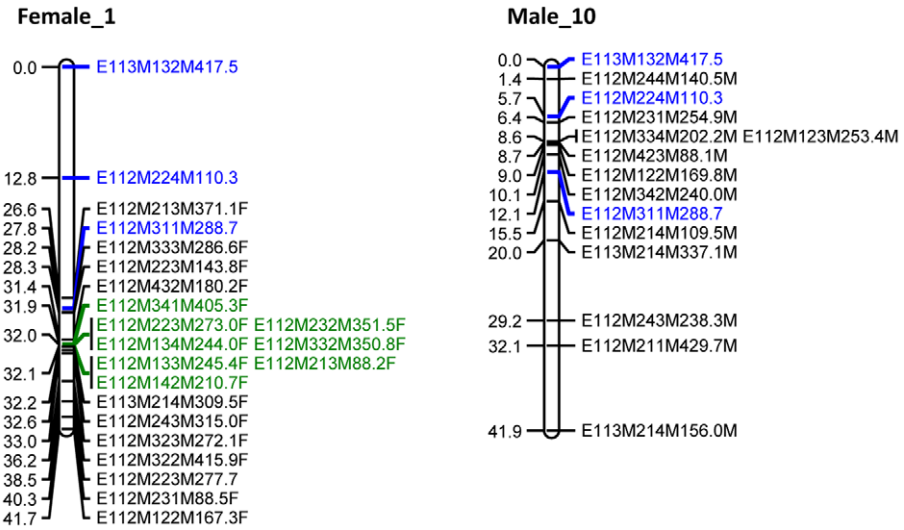

Figure 4. *Artemia franciscana* sex linkage groups. Female linkage group Female_1 corresponds with the W chromosome. The homologous male linkage group Male_10 corresponds with the Z chromosome. Each AFLP marker is represented by (1) a code referring to the corresponding PC (Table S1), followed by (2) the molecular size of the fragment in nucleotides and (3) the type of marker (female marker, tagged as "F", male marker, tagged as "M", biparental marker, no tag). Common biparental markers are indicated in blue. Markers fully linked to sex are marked in green. Cumulative marker distances (cM) are indicated on the left.
doi:10.1371/journal.pone.0057585.g004

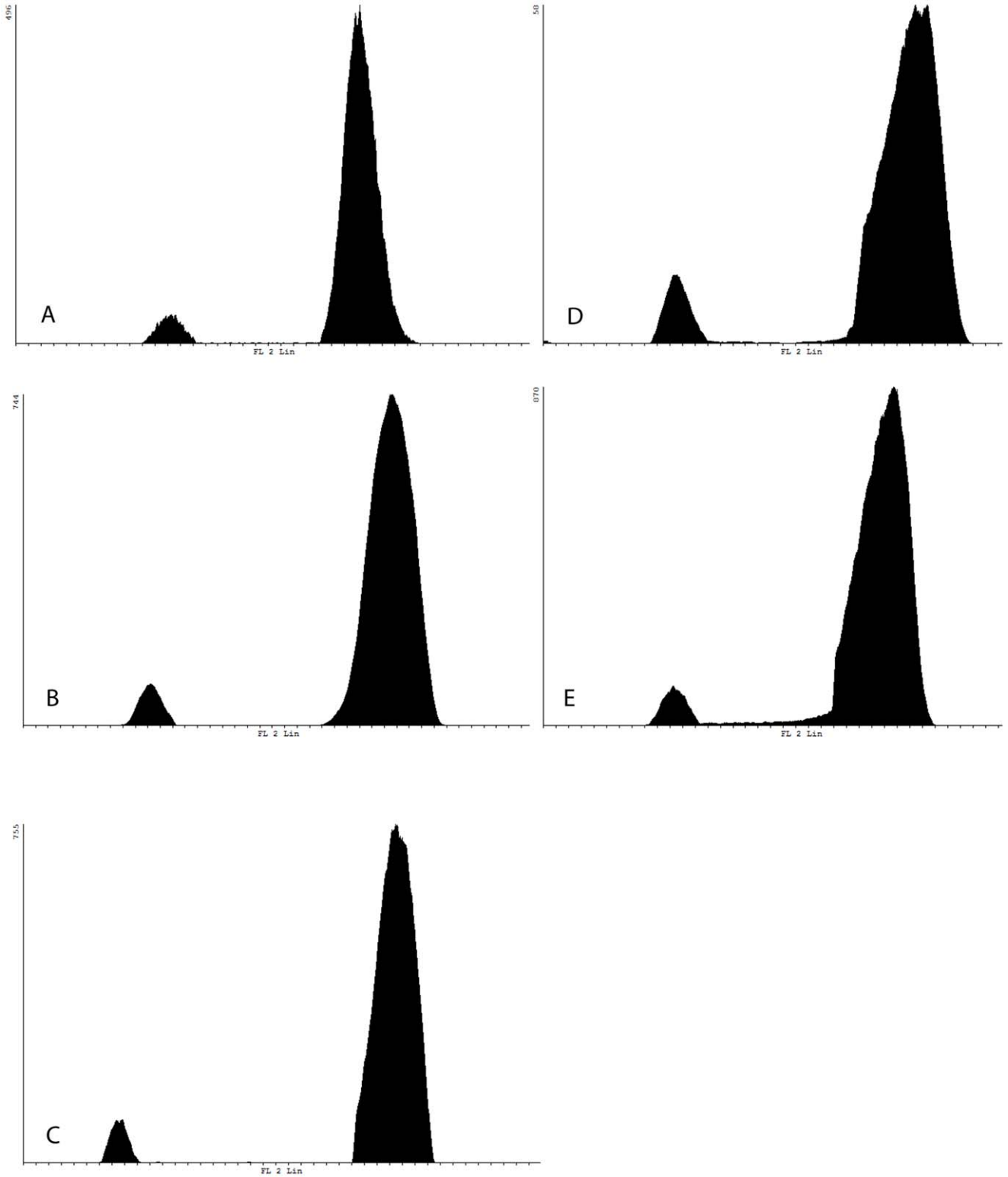

Figure 5. Fluorescence histograms for *Artemia franciscana* and chicken DNA content estimation. Fluorescence histogram of four different *A. franciscana* male individuals with trout as the internal standard (A, B, C, D) and of chicken CEN with trout as the internal standard (E).
doi:10.1371/journal.pone.0057585.g005

This will be especially useful, considering the numerous reports of repetitive sequences in *Artemia* [63]. *Artemia* linkage maps will also allow future linkage studies in *Artemia* for important crustacean traits such as resistance to *Vibrio*, the most common bacterial pathogen in worldwide marine fish and shellfish aquaculture.

Fifteen homologous linkage groups, including the LG representing the sex chromosomes, were identified between the female

and male linkage maps by including biparental markers in the linkage analysis. This study identified eight sex-linked AFLP marker alleles mapping to one locus and inherited from the female parent, suggesting *A. franciscana* adopts a genetic WZ-ZZ sex-determining system. *Artemia* sex-linked markers will enable the study of nauplii sex ratios and their dynamic in natural *Artemia* populations. They will also enable the further fine-mapping of the sex-determining locus and the subsequent identification of the primary sex-determining gene(s). Furthermore, based on sequence homology with *Artemia*, sex-determining genes might be identified in commercially valuable crustaceans, enabling PCR-based allele-specific assay development in the framework of the development of mono-sex cultures in shrimp [64].

The clustering of eight sex-linked markers in a 0.2 cM region suggests reduced recombination, which is often found in sex-linked regions [65]. Genes from a region that stopped recombining in the early evolution of sex chromosomes have a high sequence divergence, allowing an estimate of when the W and Z chromosomes first stopped recombining and thus, the age of the sex chromosome system [65].

The estimated *Artemia* GS in this study (0.93 Gb) is smaller than earlier estimates: 2.93 Gb by Feulgen densitometry [9] and 1.47 Gb by DNA reassociation kinetics [10]. "*A. salina*" used to be a general name for all *Artemia* species, presently confounding the identity of the investigated *Artemia* in many studies [1]. Because the *Artemia* DNA content measured by Feulgen densitometry on "*A. salina*" is almost a twofold of that measured by DNA reassociation kinetics, Feulgen densitometry might have been performed on a tetraploid *A. parthenogenetica*, as suggested by Vaughn [10]. Also, the absolute *A. franciscana* karyotype size varies between 60.68 µm and 139.26 µm [8], showing that significant intra-specific variation in DNA content could explain the high Feulgen densitometry values as well.

Vaughn [10] calculated the *Artemia* haploid GS by DNA reassociation kinetics, based on an *A. franciscana* GC content of 42%. More recent measurements however, show an *A. franciscana* (SFB) GC content of 32% determined by CsCl centrifugation and confirmed by direct chemical analysis and renewed thermal denaturation [61]. An estimated GC content lowered by 1% results in a 0.018% lower haploid DNA content estimated by DNA reassociation kinetics [66]. Hence, based on a GC content of 32%, the corrected *A. franciscana* DNA content estimated by Vaughn [10] is 1.23 Gb, approximating more closely the 0.93 Gb *Artemia* GS estimated in this study.

Currently, out of the 50,000 known Crustacea species, the GS of 278 crustaceans has been determined, covering a 400-fold-wide genome size range between *Cyclops kolensis*, a Cyclopoid copepod (0.14 pg) and *Ampelisca macrocephala*, an Arctic Amphipod (64.62 pg) [67,68]. In comparison, *A. franciscana* has a relatively small genome of 0.97 pg. This makes it a potential new model crustacean for which genome sequencing is currently feasible, unlike for crustaceans with a much larger genome size. To date, the only publicly accessible sequenced crustacean genome is the branchiopod *D. pulex*, with an average genome size of 0.23 pg [69].

Ultimately, the further development of genomic resources for *Artemia* such as the whole genome sequence, will add a completely new dimension to *Artemia* research and its use as live food in aquaculture. Moreover, knowledge of the *A. franciscana* sex-determining system will facilitate future evolutionary studies of sex chromosomes in sexually dimorphic (WZ female/ZZ male) and parthenogenetic *Artemia*. Considering the presence of sexual and asexual reproduction strategies, the *Artemia* genus shows promise as a model system for the study of asexuality, its evolution and its evolutionary purpose. Finally, since *Artemia* is considered a potential crustacean model species, increasing knowledge about *Artemia* genetics and genomics in general and sex-related genetics in particular, are expected to be valuable to crustacean aquaculture, presently lacking in molecular breeding strategies despite their contribution of 23% to the total aquaculture production value [70].

Supporting Information

Table S1 List of the 65 primer combinations used for AFLP analysis[1] E: *Eco*RI primer with three selective bases; M: *Mse*I primer with three selective bases (1, 2, 3, 4 correspond to A, C, G, T).
(DOC)

Acknowledgments

The authors would like to thank Christ Mahieu, for rearing the *Artemia* mapping population and Professor Annemie Decostere and Annelies Declercq, for donating rainbow trout blood.

Author Contributions

Conceived and designed the experiments: SDV PB MV. Performed the experiments: SDV IV GV. Analyzed the data: SDV MV. Contributed reagents/materials/analysis tools: PB IV PS. Wrote the paper: SDV PB GV MV PS.

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