Amplified fragment length polymorphism (AFLP) reveals species-specific markers in the *Daphnia galeata-hyalina* species complex

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Abstract

Daphnia often occur in species complexes that consist of two or more co-occurring species and their hybrids. Hybrid individuals are often capable of sexual reproduction and so backcrossing with introgression occurs. To better understand hybridization and backcrossing frequency, we sought to develop PCR-based, species-specific markers in the *Daphnia galeata-hyalina* species complex using amplified fragment length polymorphism (AFLP). This technique produces large numbers of reproducible markers for assessing diversity across the nuclear genome and provides several advantages over mtDNA and micro-satellite approaches. We examined 28 clones of *D. galeata*, *D. hyalina*, and their hybrids isolated from Lake Constance on the Swiss-German border. Using a single AFLP primer combination we found five potential species-specific markers, defined as bands that occurred in >80% of one parental species and <20% of the other. Two bands appeared to be co-dominant and were present (homozygous) in *D. galeata*, absent in *D. hyalina*, and heterozygous in the hybrid. We conclude AFLP could provide enough PCR-based, species-specific markers to identify species, hybrids, and backcrosses from even small amounts of tissue (i.e. resting eggs).

Introduction

Determining the *Daphnia* species composition of European lakes is not always an easy task. Sibling species typically co-occur and often hybridize (Schwenk & Spaak, 1995). Although attempts have been made to distinguish taxa morphologically (Flöbner, 1993; Giebler, 2001), it remains difficult for non-specialists to quantify species and hybrid frequencies. With the help of genetic techniques (allozymes), Wolf & Mort (1986) and Wolf (1987) found that certain *Got* (renamed to *AAT*) alleles were species-specific and can be used to identify hybrids and parental species within the *Daphnia galeata-hyalina-cucullata* complex. Giebler (1997) identified *AO* as another diagnostic locus, provid-

ing the possibility to distinguish backcrosses. Further studies have found that hybridization and backcrossing are common events in natural populations (Spaak, 1996; Schwenk, 1997; Schwenk et al., 1998). All studies published to date show evidence for backcrossing within the *Daphnia galeata-hyalina-cucullata* species complex, but with only two species-specific markers it remains difficult to identify individual backcrosses (but see Jankowski, 2002; Spaak et al., 2004).

Schwenk (1993) found species-specific mtDNA restriction sites, and since mtDNA is inherited maternally it can be used to investigate which species served as the maternal parent of a hybrid

individual. In a later study Schwenk (1997) found that the directionality of hybridization was non-random; *D. cucullata* was significantly more frequently the maternal species in *D. galeata-cucullata* hybrids. Other studies have used mtDNA sequence information to study species composition of ephippial eggs sampled from lake sediments (Duffy et al., 2000) or for phylogenetic studies (Colbourne & Hebert, 1996; Schwenk et al., 2000). Microsatellites have been developed for a number of *Daphnia* species (Ender et al., 1996; Pfrender et al., 2000; Limburg & Weider, 2002). Microsatellites are co-dominant and often highly variable; however, sequence knowledge is required for primer design and no species-specific loci have been reported yet for *Daphnia*.

The amplified fragment length polymorphism technique (AFLP, Vos et al., 1995) has received increasing attention for the study of hybridization. The technique produces large numbers of reproducible markers for assessing diversity across the entire nuclear genome (Fig. 1), providing several advantages over mtDNA and microsatellite approaches. No prior knowledge of the genome is

required, thus eliminating the time required for marker development. At the intraspecific level AFLP has successfully been used to differentiate populations of animals and plants (e.g., O'Hanlon et al., 1999; Bensch et al., 2002). Most studies use AFLP only as a dominant marker (e.g., Ogden & Thorpe, 2002); however, automated sequencers and software that measures band intensity enable the scoring of co-dominant markers, where the heterozygous locus is marked by a band with half the intensity of the homozygous locus (Castiglioni et al., 1999; Wong et al., 2001).

The goals of the present study were to investigate the utility of AFLP to quantify genetic variation in *Daphnia* and to identify species-specific genetic markers for *D. galeata* and *D. hyalina*. The aim was to find markers that enable the recognition of parental species, hybrids, and backcrosses. We examined 28 individuals of *D. galeata*, *D. hyalina*, and *D. galeata-hyalina* hybrids (based on their *AAT* and *AO* allozyme alleles) from Lake Constance, on the Swiss-German border, where both parental species, hybrids, and backcrosses occur together.

Figure 1. Part of an AFLP-gel with banding patterns of 10 individual *Daphnia*. One individual is enclosed with black lines. The arrows indicate the lanes in which size markers were run. The numbers indicate the size of the markers in base pairs (bp).

Material and methods

Origin of *Daphnia* clones

Daphnia clones from Lake Constance had been cultured in the laboratory for more than 1 year. We selected the clones based on the genotype for the diagnostic allozymes *AAT* and *AO*, using clones with the *D. galeata*, *D. hyalina* or the hybrid genotype for both loci (Table 1). The clones represented 17 different multilocus genotypes (MLG) based on allozyme electrophoresis for the enzymes *AAT*, *AO*, *PGI* and *PGM*.

DNA extraction

DNA was extracted using a 'salting out' procedure modified from (Sunnucks & Hales, 1996). *Daphnia* were incubated with proteinase K in 200 µl TNES buffer (50 mM Tris, 20 mM EDTA, 400 mM NaCl, 0.1% SDS) at 55°C for 2–3 h. After incubation, 25 µl of 5 M NaCl was added, followed by centrifugation at 3000 rpm for 10 min. The supernatant was transferred to new tubes and an equal volume

Table 1. Overview of the different allozyme genotypes used in this study. All clones were included in the AFLP analysis as indicated in the last columns

Taxon	AAT-AO-PGI-PGM Genotype	No. individuals	MseI-EcoRI
<i>D. gal. × hyl</i>	SF + SFMMFF	2	2
<i>D. gal. × hyl.</i>	SFSFMMFF	1	1
<i>D. gal. × hyl.</i>	SFSFMMMF	2	2
<i>D. gal. × hyl.</i>	SMSFMMFF	1	1
<i>D. gal. × hyl.</i>	SMSFMMFF+	2	2
<i>D. galeata</i>	FFFFMFFF	1	1
<i>D. galeata</i>	FFFFMFSF	1	1
<i>D. galeata</i>	FFFFMFSM	1	1
<i>D. galeata</i>	FFFFMMFF+	1	1
<i>D. galeata</i>	FFFFMMMF	2	2
<i>D. galeata</i>	MFFFMFSF	1	1
<i>D. galeata</i>	MMFFMFFF	1	1
<i>D. galeata</i>	MMFFMMMF	1	1
<i>D. galeata</i>	MMFFMMSF	1	1
<i>D. hyalina</i>	SSSSMMFF	7	7
<i>D. hyalina</i>	SSSSMMFF+	1	1
<i>D. hyalina</i>	SSSSMMMF+	2	2
	Total	28	28

of chloroform was added. Following centrifugation at 8000 rpm for 5 min, the aqueous phase was transferred to new tubes and DNA was precipitated with an equal volume of 100% ethanol (−20 °C). DNA was pelleted by centrifugation (14 000 rpm, 20 min) and washed twice with 70% ethanol before being air-dried and resuspended in 30 µl autoclaved nanopure water.

AFLP analysis

AFLP procedures, including all adapter and primer sequences, generally followed those of (Vos & Kuiper, 1997). An aliquot of total DNA (8 µl) was digested using 20 U *MseI* and 10 U *EcoRI* in NEB buffer No. 2 (New England Biolabs) with 1×BSA in a 20-µl reaction at 37 °C for 2–3 h. Ligation was started immediately by adding a 5-µl mix of *MseI* (27.5 pmol) and *EcoRI* (2.75 pmol) adapters, T4 DNA Ligase, and 1×ligation buffer. Ligation was carried out overnight at 12 °C. Preamplification PCR was performed in 20-µl volumes, with 2 µL diluted restricted/ligated DNA, 1×PCR buffer, 1.5 mM MgCl₂, 0.2 mmol of each dNTP, 30 ng *MseI* + C primer and 30 ng *EcoRI*. PCR was carried out with a Primus 96 Plus thermocycler

(MWG Biotech) with 2-min initial denaturation at 94 °C followed by 35 cycles of denaturing (94 °C 30 s), annealing (56 °C 1 min), and extension (72 °C 1 min). For selective amplification, preliminary trials using several different primer combinations gave best results using *MseI* + CAG and *EcoRI* + AC primers. Selective amplification PCR was performed in 20-µl volumes, with 1 µL pre-amplification product as template, 1×PCR buffer with 1.5 mM MgCl₂, 0.2 mmol of each dNTP, 30 ng *MseI* primer and 10 ng *EcoRI* primer. *EcoRI* primers were labelled (3' end) with IRD700 (MWG Biotech) infrared dye. PCR consisted of 2 min denaturation (94 °C), 13 cycles of 30 s 94 °C, 30 s 65 °C, and 1 min 72 °C, with the annealing temperature decreasing from 65 °C by 0.7 °C increments in cycles 2–13. This was followed by 26 cycles of 30 s 94 °C, 30 s 56 °C, and 1 min 72 °C.

PCR products from the selective amplification were denatured with microSTOP loading buffer (Microzone Limited, East Sussex, UK). Electrophoresis and band/fragment detection was carried out on a Li-Cor 4200 LongReadIR automatic sequencer. Electrophoresis was run at 45 W (~1500 V), 40 mA, and 45 °C, using 6% polyacrylamide gels and 41-cm plates. Fragment sizes (bp)

were determined using microSTEP 24a (Microzone Limited) fluorescent marker and Gene ImagIR v 4.03 (Scanalytics) software.

Data analysis

The AFLP bands we scored were dark, consistent and repeatable in individuals across PCR reactions and gels. We scored 75 bands (AFLP restriction sites), ranging from 70 to 307 bp in length. Of the 75 bands, 71 were polymorphic and 12 bands occurred in only one individual. Band presence/absence was scored in a binary data matrix and a dendrogram was constructed with the complete linkage method using Statistica (StatSoft, 2001) using the dissimilarity between the clones. A similarity matrix, constructed using the Jaccard coefficient (Sneath & Sokal, 1973), was used for a multidimensional scaling analysis (MDS) using Statistica.

Potential species-specific bands should be indicated by a high frequency in one parental species and the hybrid and a low frequency in the other parental species. Ideally bands would occur in 100% and 0% frequencies, but because (1) backcrossing occurs in this species complex, and (2) the clones we examined naturally co-occur with hybrids and parentals, we cannot exclude the occurrence of introgression in our laboratory-cultured clones. Therefore we used a criterion of 20%. If a band had a frequency above 80% in both the hybrid and a parental species and a frequency below 20% in the other parental species it was considered as a potential species-specific marker.

Results

Species-specific markers

Two bands (104, 210 bp) were present in all *D. hyalina* and occurred in only 10% (104 bp) or were absent (210 bp) from *D. galeata* clones (Table 2). Both of these bands occurred in all hybrids (Table 2), making them strong candidates for species-specific markers in *D. hyalina*. Three bands (166, 189, 190 bp) occurred in $\geq 80\%$ of *D. galeata* and $\leq 20\%$ of *D. hyalina* (Table 2), but all three bands were present in 62.5 or 75% of hybrids. This was lower than our 80% criterion set *a priori* for species-specific markers, but suggests the presence of

species-specific bands masked by backcrossing and introgression that could be distinguished with additional sampling.

Genetic variation

The UPGMA dendrogram revealed two primary clusters, one with all but a single *D. galeata* clone (as defined *a priori* by their allozyme pattern for *AAT* and *AO*) and one with *D. hyalina* and the hybrids. This second cluster was further divided into a *D. hyalina* cluster with three hybrid clones and a hybrid cluster with 1 *D. galeata* clone.

The two-dimensional plot generated from multidimensional scaling of the Jaccard index gave slightly more pronounced separation of parentals and hybrids (Fig. 3). *D. galeata* clones clustered together and were distinctly separated from the cluster of *D. hyalina*. The two-dimensional area of the clusters indicated genetic variation was similar for both species. Five of the hybrid clones clustered intermediate of the parental species and two hybrids clustered within the *D. hyalina* cluster. A single individual (X08) did not cluster with any others.

Co-dominance of AFLP bands

Two of the potential *D. galeata*-specific bands (166, 189 bp) appeared co-dominant, with nearly all hybrids exhibiting less intensity (i.e., fainter bands; Fig. 4) than the parental. The 166-bp band (see Fig. 4) was present but faint in one *D. galeata* and in one *D. hyalina* individual, suggesting they could be backcrosses (see Discussion). The 189-bp fragment was present but faint in two *D. galeata* and one *D. hyalina*, also suggesting the presence of backcrosses. Similar patterns were observed for the two *D. hyalina*-specific bands (104, 210 bp), with hybrids exhibiting reduced band intensity.

Discussion

Distinguishing among parental species and hybrids

Our results show that with AFLP analysis a clustering of taxa within the *D. galeata*–*hyalina* species complex can be achieved that is comparable to that achieved with allozymes. The *Daphnia* clones

Table 2. Frequency of the 20 most frequently occurring bands, sorted by each of the three taxa examined

<i>D. hyalina</i> sorted				Hybrid sorted				<i>D. galeata</i> sorted			
<i>n</i> = 10				<i>n</i> = 8				<i>n</i> = 10			
MW	<i>D. galeata</i>	<i>D. gal.</i> × <i>hyl.</i>	<i>D. hyalina</i>	MW	<i>D. galeata</i>	<i>D. gal.</i> × <i>hyl.</i>	<i>D. hyalina</i>	MW	<i>D. galeata</i>	<i>D. gal.</i> × <i>hyl.</i>	<i>D. hyalina</i>
77	1.00	1.00	1.00	77	1.00	1.00	1.00	77	1.00	1.00	1.00
139	1.00	1.00	1.00	139	1.00	1.00	1.00	113	1.00	0.75	1.00
146	1.00	1.00	1.00	146	1.00	1.00	1.00	139	1.00	1.00	1.00
223	1.00	1.00	1.00	191	1.00	1.00	0.90	146	1.00	1.00	1.00
91	0.90	1.00	1.00	223	1.00	1.00	1.00	162	1.00	0.88	0.80
210	0.10	1.00	1.00	70	0.90	1.00	0.90	166	1.00	0.63	0.20
104	0.00	1.00	1.00	91	0.90	1.00	1.00	191	1.00	1.00	0.90
224	1.00	0.88	1.00	137	0.90	1.00	0.80	223	1.00	1.00	1.00
95	0.80	0.88	1.00	210	0.10	1.00	1.00	224	1.00	0.88	1.00
158	0.70	0.88	1.00	104	0.00	1.00	1.00	70	0.90	1.00	0.90
113	1.00	0.75	1.00	162	1.00	0.88	0.80	91	0.90	1.00	1.00
114	0.90	0.75	1.00	224	1.00	0.88	1.00	114	0.90	0.75	1.00
225	0.10	0.63	1.00	90	0.80	0.88	0.70	137	0.90	1.00	0.80
191	1.00	1.00	0.90	95	0.80	0.88	1.00	189	0.90	0.63	0.10
70	0.90	1.00	0.90	158	0.70	0.88	1.00	190	0.90	0.75	0.10
252	0.00	0.75	0.90	145	0.60	0.88	0.70	90	0.80	0.88	0.70
137	0.90	1.00	0.80	177	0.30	0.88	0.60	95	0.80	0.88	1.00
162	1.00	0.88	0.80	113	1.00	0.75	1.00	158	0.70	0.88	1.00
106	0.10	0.63	0.80	114	0.90	0.75	1.00	232	0.70	0.63	0.50
307	0.00	0.38	0.80	190	0.90	0.75	0.10	285	0.70	0.00	0.00

Bold bands indicate possible species-specific alleles based on our 20% criterion, where bands with a frequency above 80% in both the hybrid and a parental species and a frequency below 20 in the other parental species are defined as a potential species specific marker. MW (molecular weight) indicates fragment size.

that we classified as *D. hyalina* and *D. galeata* using the *AAT* and *AO* loci clustered in different groups (Figs. 2 and 3). Both UPGMA and multi-dimensional scaling results strongly suggest AFLP can be used to differentiate taxa in *Daphnia* species complexes as has been demonstrated in vertebrates (e.g., Bensch et al., 2002) and plants (e.g., Beismann et al., 1997). The fact that only one *D. galeata* clone occurred outside the *D. galeata* grouping (UPGMA, Fig. 2) and all *D. galeata* clustered together with multidimensional scaling (Fig. 3) suggests that our *a priori* designation of taxon, using two species-specific allozyme markers, probably excluded most of the backcrosses that are likely present (Spaak, 1996; Gießler et al., 1999).

One of the candidate species-specific bands (104bp) occurred in all parental *D. hyalina*, all hybrids, and in no *D. galeata*. While this is the

ideal case and therefore the best candidate for being species-specific, data from more individuals and more lakes are required for confirmation. The other four potential species-specific bands all occurred in at least some of the other parental species. This requires additional study, largely because of the occurrence of backcrossing in Lake Constance (Jankowski, 2002). When F1 or F2 hybrids backcross with a parental, the number of species-specific markers needed for correct identification increases to 5 or more markers (Boecklen & Howard, 1997). However, the results from only a single AFLP primer pair (MseI + CAG/EcoRI + AC) are encouraging, having produced five potentially species-specific bands. We also consider that our criterion for a species-specific band (>80% in one species, <20% in the other) is somewhat conservative. For example, Bensch et al. (2002) used the criterion that ‘markers should be at

Figure 2. Dendrogram of AFLP band-sharing dissimilarity for the 28 *Daphnia* clones. The first letter of the clone name indicates the taxon (G = *D. galeata*; H = *D. hyalina*; X = interspecific hybrid).

least three times as common in one of the taxa than in the other' and be present in at least 23.3% of individuals in the taxon with the highest frequency of that marker (Bensch et al., 2002).

Our results make clear that the search for species-specific markers ideally would use clones from populations where the parental species do not co-occur. The use of *D. hyalina* clones from Lake Constance was, in our opinion, justified since Lake Constance is the type location for *D. hyalina* (Leydig, 1860).

Co-dominance

Several recent studies (Castiglioni et al., 1999; Piepho & Koch, 2000; Jansen et al., 2001; Wong

et al., 2001) have reported co-dominance of AFLP restriction sites. Individuals heterozygous for an AFLP locus exhibit bands that have half the intensity of homozygous bands. Applying this to *Daphnia* hybrids, we found that hybrids exhibited 166 and 189 bp bands at lower intensities than parental *D. galeata* (Fig. 4). This suggests that the parental has two copies and the hybrid only one copy of the restriction site as has been shown previously (e.g., Piepho & Koch, 2000; Wong et al., 2001). Since low amounts of template DNA and sub-optimal PCR conditions also affect band intensity, standardized DNA template volumes and PCR conditions are necessary steps in using species-specific co-dominant bands to identify hybrids. Another important consideration for

Figure 3. Two-dimensional multidimensional scaling plot of Jaccard distances calculated using the AFLP presence/absence dataset. The clones from both parental species (as defined by allozyme electrophoresis) cluster together: group A *D. hyalina*; group B contains the *D. galeata* clones. Most hybrid clones appear intermediate to the clusters, although 2 occur within the *D. hyalina* cluster.

co-dominance in AFLP is that a band with relatively low intensity for a homozygote might not be detectable for a heterozygous individual. It is therefore more likely that species-specific bands are not detected in hybrids compared to the parental species.

In summary, our data are limited, but the fact that hybrids clustered within the *D. hyalina* cluster and not within the *D. galeata* cluster suggests that hybrids backcross more frequently with *D. hyalina*

than with *D. galeata*, as has been observed in previous studies (Jankowski, 2002). AFLP hold much promise for the delineation of hybrid-species complexes, especially for small, soft-bodied invertebrates for which the small tissue requirements of PCR techniques are well-suited. This is particularly true for *Daphnia*, which often are too small to examine for 5 or more allozyme loci that would be required to determine F1 and F2 backcross frequencies.

Figure 4. Fragment of AFLP gel showing the banding pattern of 28 *Daphnia* clones from Lake Constance. The lanes marked with L are the size marker ladder with the 160, 180 and 200 bp markers. The bands of interest are marked with two arrows. The 166 and 189 bp bands show a high intensity for the *D. galeata* (G) clones, a low intensity for most hybrid clones (X) and are absent for almost all the *D. hyalina* (H) clones. The numbers in each lane indicate the intensity of the bands on a 0–100 scale. The two shaded lanes are excluded from the analysis because almost no bands were detected.

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