MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2010) **10**, 324–330 doi: 10.1111/j.1755-0998.2009.02762.x

TECHNICAL ADVANCES

Semi-quantitative differences in gene transcription profiles between sexes of a marine snail by a new variant of cDNA-AFLP analysis

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Abstract

A variant of the cDNA-AFLP method coupled to an automated sequencer was used to quantify transcripts differentially expressed between sexes of the marine snail *Littorina saxatilis*. First, we conducted a validation study of the technique using known concentrations of a commercial marker. Second, we analysed six replicates of males and females from a population showing no apparent sexual dimorphism. The results confirm that the method can be properly used within the range of DNA concentrations utilized. In addition, we detected a small percentage of spots (1.8%) differentially expressed between sexes, as expected from a low to moderately sexual dimorphic species.

Keywords: cDNA polymorphism, expression differences, gene expression, sex differences, sexual differentiation, transcriptomics

Received 22 April 2009; revision accepted 20 July 2009

Introduction

Transcriptomics, also called genomic-wide expression profiling, is an important tool that can help us obtain a better understanding of genes and pathways involved in various biological processes. As transcription is the first step in gene regulation, information from this level allows us to identify genes that are being actively expressed as well as gene regulatory networks (Gomase & Tagore 2008). Increasingly, studies have reported variation in gene expression at this level both within and among populations in relation to adaptation (Gibson 2002; Bochdanovits *et al.* 2003; Ranz *et al.* 2003; Whitehead & Crawford 2005; Derome & Bernatchez 2006; Derome *et al.* 2006, 2008; Baker *et al.* 2007; Roberge *et al.* 2007). These works improve our knowledge of the molecular mechanisms underlying the process of adaptation, population divergence and ultimately speciation (Derome *et al.* 2008). Moreover, expression studies are also of interest in studies of sex differences in natural populations. Thousands of genes have been identified as differentially expressed in the gonads of many species (Parisi *et al.* 2004; Small *et al.* 2005; Santos *et al.* 2008), and although less pronounced than in gonads (Santos *et al.* 2008), an increasing amount of data also demonstrates sexual dimorphism in somatic tissues from nematodes, insects, fish, birds and mammals (Boag *et al.* 2000; Meyer *et al.* 2005; Yang *et al.* 2006; Baker *et al.* 2007; Désert *et al.* 2008; Ehmann *et al.* 2008). However, little effort has been devoted to research the role of sex in the context of an adaptive radiation (Pröschel *et al.* 2006) or the evolutionary consequence of sex linkage of genes involved in speciation (Servedio & Saetre 2003).

One shortcoming of microarray technology is that it requires prior knowledge of species-specific sequences and, although more and more sequence information has become available in the past few years, sequence information for less well-characterized species remains limited. Furthermore, the availability of whole genome sequences is, in itself, not sufficient because of the complicated annotation.

Fortunately, gene expression systems for which no pre-existing biological or sequence information is needed are also available, and these can be applied to a much

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wider range of species. These also inherently have the advantage of simultaneously identifying and assessing new genes (Reijans *et al.* 2003). One such system, cDNA-AFLP, has the advantage of high reproducibility and sensitivity (Bachem *et al.* 1996), high-throughput genome-wide capability (Breyne & Zabeau 2001), low set-up cost (Donson *et al.* 2002) and the fact that it does not require prior sequence information (Ditt *et al.* 2001).

The suitability of the cDNA-AFLP technique for quantitative expression profiling has long been recognized in the use of both radioactive isotopes and silver staining in conventional polyacrylamide gel electrophoresis (e.g. Bachem *et al.* 1996; Breyne *et al.* 2003; Reineke *et al.* 2003; Gigliotti *et al.* 2004; Knight *et al.* 2006; Wee *et al.* 2008; Xiao *et al.* 2009). However, to our knowledge, only two studies have contemplated using fluorescent dyes (Metsis *et al.* 2004; Vuylsteke *et al.* 2007). In this study, we have applied a new variant of cDNA-AFLP using fluorescently labelled primers coupled to an automated sequencer to quantify transcripts which are differentially expressed, thanks to the normalization method provided by GeneMapper® software. This approach can help to (i) increase sensitivity, (ii) improve scoring efficiency, and (iii) reduce the rate of false positive peaks as a result of the co-migration of fragments of very similar sizes. In addition, this method avoids hazardous radioactive methods and offers higher sensitivity than silver staining.

Our main objective was to test the usefulness of this method in assessing putative sex differences in *Littorina saxatilis*. This marine snail has been claimed to be an example of the putative sympatric ecological speciation process (Rolán-Alvarez 2007). This species shows little or no sexual dimorphism in most populations (Reid 1996), but in certain populations, females can be considerably larger than males (Johannesson *et al.* 1995) or have slightly different shapes (Grahame & Mill 1992). In addition, the species shows chromosomal sex determination, with the male XY and the female XX (Rolan-Alvarez *et al.* 1996). It is therefore plausible that some sex-specific transcriptional regulation exists.

Materials and methods

Sample collection and preparation

Adult males and females of *Littorina saxatilis* (from upper shores) were collected from Silleiro (NW Spain; 42°6.17′8″N; 8°53′56″W) in July 2006. Snails were carried to the laboratory immediately after sampling and sexed according to the presence of a penis in males and a brood pouch of shelled embryos in females, ensuring that the individuals were sexually mature. We prepared six replicate samples of each sex (hereafter referred as biological replicates), each including 10 pooled male (or female) specimens, for a total of 120 animals. The shelled embryos of each female were discarded from the pools.

RNA extraction and cDNA synthesis

Total RNA was isolated from the pools using TRIZOL® Reagent according to the manufacture's instructions. High quality starting RNA is essential for the cDNA-AFLP technique. Therefore, to assess the integrity of the total RNA, an aliquot of each sample was run on agarose gel. Moreover, the concentration and purity (i.e. the A_{260}/A_{280} ratio) of each RNA sample were checked with an UV spectrophotometer (UNICAM UV/Vis UV2). Next, the Turbo DNA-free™ kit (Ambion) was used to remove any remaining contaminating DNA from the total RNA extractions. The concentration was measured again by spectrophotometry. Finally, cDNA was synthesized from 25 μg of total RNA using the SuperScriptTM Double-stranded cDNA Synthesis Kit (Invitrogen) and a biotinylated oligo $(dT)_{18}$ primer.

cDNA-AFLP analysis

cDNA-AFLP analysis represents a modification of the Vos *et al.* (1995) method for the analysis of AFLP fragments. The restriction enzymes used were *Taq*I and *Mse*I, each with a 4-bp recognition sequence, following previous studies using cDNA (Habu *et al.* 1997; Reineke *et al.* 2003). The digestion was performed in two separate steps. Briefly, 500 ng of cDNA was first digested with *Taq*I for 4 h at 37 °C. The enzyme was subsequently inactivated by heating for 10 min at 70 °C. After digestion with *Taq*I, the 3′-end fragments with biotinylated tails were collected by streptavidin magnetic beads using the PolyATract® Systems III kit (Promega). The cDNA fragments on the magnetic beads were digested with *Mse*I for 4 h at 37 °C. The supernatant including the digested fragments was collected and used as a template in the subsequent AFLP steps, whereas the 3′-end tails that remained bound to the beads were discarded. This process allowed the collection of more informative tag fragments by eluting the long polyA tails, as well as lowering the redundancy of the fragments obtained from each messenger. *Mse*I and *Taq*I adaptors were ligated for 16 h at 16 °C in a total volume of 50 μL. The *Mse*I and *Taq*I adaptors were prepared by mixing equimolar amounts (50 pmol each) of the oligonucleotides 5′-GACGATGAGTCCTGAG-3′ and 5′-TACTCAGGACTCAT-3′ for *Mse*I adaptor and 5′-GACGATGAGTCCTGAG-3′ and 5′-CGCTCAGGACTCAT-3′ for *Taq*I adaptor. Pre-amplification of cDNA fragments was performed for 20 cycles with a 4-μL aliquot of a 1:10 dilution of the ligation reaction in a total volume of 20 μL, using 20 pmol of the

primers corresponding to the *Mse*I and *Taq*I adaptor sequence without an extension (*Mse*I primer 5′-GATGAGTCCTGAGTAA-3′, *Taq*I primer 5′-GATGAGTCCTGAGCGA-3′). The 20 cycles of polymerase chain reaction (PCR) were performed at 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min. The pre-amplified products were diluted at a ratio of 1:10, and a 4-μL aliquot was selectively amplified in a total volume of 20 μL with *Mse*I and *Taq*I primers having one selective base extension at the 3′-end. Amplification included a touchdown phase in 10 cycles of PCR (94 °C for 20 s, 66 °C for 30 s and 72 °C for 2 min; annealing temperature was decreased 1 °C every cycle), followed by 20 cycles (94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min). A total of 12 primer combinations were used for selective amplifications. *Taq*I selective primers were fluorescently labelled with different dyes (6-FAM, HEX and NED).

Analysis of cDNA expression

The selectively amplified fragments were run on an ABI Prism 310 Genetic Analyzer with an internal ROX-labelled sizing ladder (Applied Biosystems). AFLP profiles were visualized and analysed using GeneMapper® v.3.7 software (Applied Biosystems), within a fragment-length (size) range of 75–500 bp. To eliminate background noise, a DNA fragment was considered to be valid if it had a peak height of at least 50 relative fluorescent units (RFU) and a ±2 base size difference with the nearest DNA fragment peak. Each AFLP expression profile was normalized using the sum of signal method as implemented in GeneMapper, to correct for differences in total electropherogram intensities that may arise because of loading errors or differences in amplification efficiency. The software sums the signals, calculates the average for all samples and then calculates the normalization factor for each sample as the ratio of the sample's sum over the average. Thus, this is a semi-quantitative analysis whereby intensity represents a normalized height (GeneMapper® v.3.7 manual). All AFLP reactions were repeated twice (each reaction representing a technical replicate) to allow the evaluation of the reproducibility of the method; the reproducibility was calculated by the Pearson correlation coefficient using these technical replicates over fragments (i.e. comparing the expression levels of the spots for the two technical replicates in each sample and averaging the coefficient across samples). We used the whole set of 24 samples (12 biological samples, each one with two technical replicates) to choose exclusively the fragments that were present in 90% of the replicates. When a fragment was detected in only one of the two technical replicates, we assigned the threshold limit value of 50 RFU to the replicate with the missing peak height.

Validation of the automated sequencer

We tested the ability of an automated sequencer to visualize differential peak heights in response to variable amounts of starting fluorescently marked DNA fragments. For this purpose, different concentrations of the ROX-labelled sizing ladder were run in three different replicates across several days.

GeneScan™ 500 ROX™ Size Standard is designed for sizing DNA fragments in the 35–500 nucleotides range, and provides 16 single-stranded labelled fragments of: 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 nucleotides (Applied Biosystems). The manufacturer's recommended loading is 4 fmol, so we ran two extra dilutions upwards and downwards from the recommended dilution (1, 2, 4, 8 and 16 fmol), to test whether the automated sequencer could detect these differences in the starting amount of DNA. This procedure also allowed us to estimate the shape of the relationship between peak height and the amount of DNA.

Statistical assessment of gene expression differences

Only common fragments (present in at least 90% of the biological replicates) were considered in the comparison among sexes. Quantitative transcript differences among sexes were assessed separately for each fragment using a nested analysis of variance (ANOVA) on normalized intensity data, with the biological replicate as a random factor nested within the factor sex (with male and female treatments) and the technical replicates as residual error. Significant cases were confirmed using a randomization ANOVA, which is very robust to deviations from normality and homoscedasticity (see Peres-Neto & Olden 2001). The Benjamini & Hochberg (1995) method was used to correct for multiple hypotheses testing using the freeware software SGOF (http://webs.uvigo.es/acraaj/SGoF.htm). Parametric nested ANOVA was carried out with the SPSS/PC version 14, and the randomization ANOVA was performed using the freeware software ANOVA (http://webs.uvigo.es/c03/webc03/XENETICA/XB2/software.htm).

Results

Validation of the automated sequencer

Table 1 shows the average peak heights of the sizing ladder across technical replicates and their standard errors for each fragment size. Peak heights were highly reproducible among technical replicates with a Pearson correlation coefficient of 0.99. Peaks steadily increased in height with higher starting amounts of fluorescently labelled DNA at all fragment sizes. A logarithmic

Table 1 Mean peak height and standard errors ($M \pm SE$) for different fragment sizes (75–500) of the DNA marker at distinct concentrations (1–16 fmol). The fragment size is in base pairs. The regression coefficient (Beta) of peak height on fragment concentrations and their percentage of variance (%), explained by the model ($100 \times r^2$), are also given for each fragment size

Size	16 fmol ($M \pm SE$)	8 fmol ($M \pm SE$)	4 fmol ($M \pm SE$)	2 fmol ($M \pm SE$)	1 fmol ($M \pm SE$)	Beta	%
75	2458 ± 18.8	2216 ± 26.3	1685 ± 19.9	1171 ± 11.0	391 ± 19.4	0.98***	97
100	2721 ± 30.7	2473 ± 58.5	1863 ± 26.0	1279 ± 16.0	424 ± 32.8	0.98***	97
139	3019 ± 30.7	2747 ± 48.6	2089 ± 4.9	1436 ± 23.7	438 ± 44.8	0.98***	96
150	3049 ± 27.9	2810 ± 54.1	2111 ± 12.8	1440 ± 28.6	434 ± 46.7	0.98***	96
160	3060 ± 31.8	2837 ± 45.3	2138 ± 16.5	1453 ± 16.7	436 ± 48.3	0.98***	96
200	3009 ± 13.7	2764 ± 62.8	2110 ± 25.5	1442 ± 16.6	419 ± 46.1	0.98***	96
250	2882 ± 22.1	2642 ± 36.4	2018 ± 20.4	1376 ± 25.8	406 ± 42.3	0.98***	96
300	2754 ± 23.0	2524 ± 34.9	1943 ± 8.7	1340 ± 18.9	394 ± 38.7	0.98***	96
340	2654 ± 22.5	2435 ± 40.3	1899 ± 10.2	1313 ± 22.7	390 ± 36.8	0.98***	95
350	2719 ± 27.3	2505 ± 34.5	1949 ± 13.9	1345 ± 13.6	396 ± 36.0	0.97***	95
400	2685 ± 19.1	2456 ± 31.4	1920 ± 3.9	1346 ± 19.8	411 ± 39.2	0.98***	95
450	2636 ± 6.3	2426 ± 28.3	1899 ± 8.7	1330 ± 23.1	407 ± 30.9	0.97***	95
490	2589 ± 11.2	2389 ± 25.7	1890 ± 9.5	1320 ± 24.3	407 ± 33.3	0.97***	95
500	2594 ± 17.9	2395 ± 20.6	1873 ± 4.6	1317 ± 14.6	401 ± 30.2	0.97***	95

***$P < 0.001$.

regression of peak height against fragment size provides a better fit to the data than a linear regression, explaining 97–98% ($P < 0.001$) of the observed variation in peak height (Table 1). This value drops to 70% when data are fitted to a linear regression (Beta = 0.83). The overall trend obtained by averaging the peak heights of all fragment sizes assayed for the same amount of starting DNA shows a logarithmic relationship between peak height and DNA concentration (Beta = 0.97; $r^2 = 0.93$; $P < 0.0001$) (Fig. 1). These results confirm the reliability of using of an automatic sequencer, at least for the range of DNA concentrations used in this experiment.

Comparing expression profiles between sexes

The extraction average of total RNA by each pool was 3.04 ± 0.15 μg/μL and the purity average 1.92 ± 0.007 indicating, a good quality of starting RNA. After the complete process, a total of 2461 fragments were scored. We assessed the variation among technical replicates for the whole set of biological samples to test the reproducibility of the cDNA-AFLP technique when used in combination with the 3100 automated ABI DNA sequencer. The Pearson correlation coefficient between technical replicates was 0.86 ($P \leq 0.05$) for the presence/absence of AFLP bands. All the subsequent quantitative data analyses were based on the subset of 168 fragments that were present in at least 90% of the biological and technical replicates. The average across specimens of the correlation (across fragments) between the two technical replicates was 0.7 ($P \leq 0.05$) for peak height intensities, increasing to 0.91 when using exclusively the seven significant fragments from transcripts identified below.

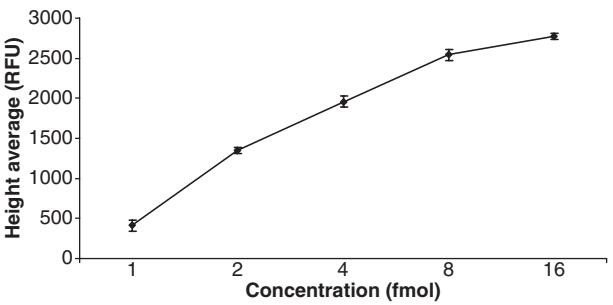

Fig. 1 Twofold serial dilutions of size standard concentrations were run from 1 to 16 fmol using three technical replicates for different fragment sizes. The points represent the mean (and standard deviation) of the replicates for the different concentrations.

The nested ANOVA allowed the identification of seven out of 168 potential single-locus diagnostic markers differing in expression between sexes. The fold change differences ranged from 1.9 to 10.6 (Fig. 2). Moreover, all of them remained significant when using a more robust nonparametric randomization ANOVA (including the technical replicates; Table 2). However, when a multi-test adjustment [maintaining the false discovery rate (FDR) at 5%] was applied to the randomized probabilities, only three out of 168 (1.8%) remained significant.

Discussion

The main advantageous feature of cDNA-AFLP is that prior sequence information is not required. In addition, the PCR-based technique, as used in this study, is considered more sensitive than hybridization-based techniques (Hoheisel & Vingron 2000), particularly when cross

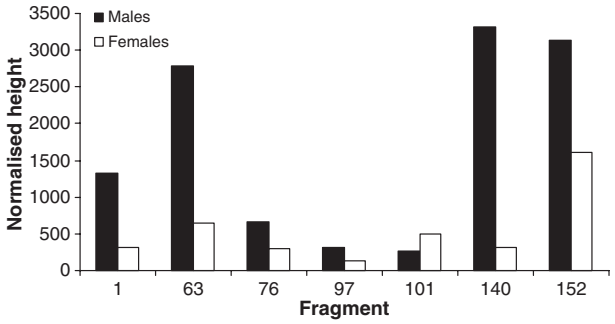

Fig. 2 Applying a nested ANOVA, 4.2% of the studied fragments showed differences in the messenger expression between sexes. Moreover, after multi-test statistical correction, three of them were still significant (fragments 1, 76 and 140).

hybridization, the major source of aberrant results in microarray data, is present (Reijans *et al.* 2003). Consequently, the method has found widespread use for gene discovery on the basis of fragment detection and for quantitative gene expression analysis (Vuylsteke *et al.* 2007). Moreover, cDNA-AFLP combines features of high-throughput analysis with the detection of rare expressed transcripts (Reijans *et al.* 2003). In this study, we present a new variant of the original method based on the visualization of differences in band intensities from conventional gels. Our purpose was to combine the use of an automated sequencer together with the GeneMapper software, commonly used in traditional AFLP studies, to carry out a semi-quantitative analysis. In this way, our method is faster, more useful, more precise and more reliable for analysing the cDNA-AFLP fingerprints than bands intensities from gels.

Moreover, with this new variant, it is also possible to gain additional information about the genes behind the fragments, for example, by running the sample in a gel to cut the band of known size, and then cloning and sequencing it (Sambrook & Russell 2001).

The first step consisted in testing the power of the automated sequencer to detect differences based on the starting amount. Our results showed that heights in the peaks increase exponentially with the starting amount of DNA, reaching a plateau at higher concentrations. Moreover, peaks were highly reproducible (99%) confirming the reliability of using the automatic DNA sequencer for quantitative cDNA-AFLP expression profiling.

We then used this technique to document transcriptomic differences between males and females in one population of the marine snail *Littorina saxatilis*. Previous studies have found a wide range of results when comparing sexes. In the nodule worm (a parasitic nematode), 39% of expressed sequence tags were expressed in a sex-specific manner (Boag *et al.* 2000). In *Drosophila* spp, 53% of genes showed sex-biased expression at the FDR of 0.05 (McIntyre *et al.* 2006). However, the percentage of differences was only 3% in the auditory brainstem of the rat (Ehmann *et al.* 2008), and 0.13% in the total brain of the adult mouse (Yang *et al.* 2006). In our case, we found a situation closer to the rat, as only 1.8% of the transcripts were differentially expressed between males and females. However, it is noteworthy that most of the above studies did not apply multi-test adjustments and so their percentages could be biased upwards. The low percentage of transcripts being differently expressed between sexes is congruent with the low sexual (phenotypic) dimorphism observed between them in these upper shore populations (see Johannesson *et al.* 1995). These differences could be associated with specific genes related to gonad differentiation or may perhaps correspond to slight differences in behaviour, as it is known that males tend to move longer distances than females when searching for mates (Conde-Padín *et al.* 2009).

Admittedly, however, the number of replicates used in this study was relatively low and it is likely that a larger sample size would have led to the detection of a higher proportion of differentially expressed bands between sexes. Nevertheless, the significant markers become interesting candidate genes to sequence and identify in the future, as the chance of detecting false positives is considerably reduced by the multiple statistical adjustments. It is noteworthy that all significant fragments except one revealed a higher level of expression in males than females (Table 2), as was previously reported in *Drosophila* (Baker *et al.* 2007). Furthermore, analysis of the functional significance of genes with transcriptional variation in the brain of the zebrafish indicated that protein synthesis was potentially more active in the male than in the female brain (Santos *et al.* 2008). In summary, our results show that the cDNA-AFLP profiling on an

Table 2 Mean peak height and standard errors ($M \pm SE$) per sex (males and females) for fragments showing significant differences by ANOVA. The ratio of the higher to the lower value, the observed *F*-value for differences between sexes and the probability of observing a larger value than the observed one after 10 000 randomizations (*P*) are presented

Fragment	Males ($M \pm SE$)	Females ($M \pm SE$)	Variation (ratio)	*F*-value	*P*-value
1	1325 ± 253.8	318 ± 18.7	+4.2	15.64	0.0001*
63	2782 ± 775.5	648 ± 190.1	+4.3	7.14	0.0043
76	667 ± 137.2	301 ± 53.1	+2.2	6.21	0.0002*
97	323 ± 69.9	141 ± 33.8	+2.3	5.48	0.0088
101	264 ± 59.7	498 ± 79.2	−1.9	5.58	0.0455
140	3326 ± 924.9	314 ± 78.8	+10.6	10.52	0.0001*
152	3138 ± 451.1	1610 ± 503.5	+1.9	5.11	0.0044

*Significant after multi-test adjustment.

automated sequencer allows for the accurate and efficient detection of significant expression differences, as observed in this study between sexes in the marine snail *L. saxatilis*.

Acknowledgements

The authors would like to thank Teresa Muiños, Pilar Alvariño and Nieves Santamaría for technical assistance, and Robert St-Laurent (Sequence detection systems and genetic analysis for Canada, Applied Biosystems Inc. (ABI)) for advice and assistance with the analysis method. We would also like to thank the MEC (CGL2008-00135/BOS) and the XUNTA de Galicia for funding. M.M.-F. is also grateful to the MEC for a FPI grant and to Dr Juan Galindo for helping with the experimental procedure.

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