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High proteome variation between ecotypes of *Littorina saxatilis* cannot be explained by tissue heterogeneity or a common-garden × ecotype effect

Angel P. Diz [a,*], Emilio Rolán-Alvarez [a,b]

[a] Department of Biochemistry, Genetics and Immunology, Faculty of Biology, University of Vigo, 36310 Vigo, Spain
[b] Department of Ecology and Evolutionary Biology, University of California, Irvine 92697, CA, USA

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ABSTRACT

In small organisms where gene expression analyses are accomplished on whole specimens rather than individual tissues, the differences observed in gene expression levels between groups of samples are assumed to be caused by regulatory differences in gene expression within tissues. An alternative explanation is the lack of homogeneity distribution of the different tissues across groups of samples. In the case of the marine snail *Littorina saxatilis*, previous proteomic studies suggested a high differentiation in protein expression between the two (RB and SU) sympatric ecotypes existing on exposed rocky shores of the Galician coast (NW of Spain). As these ecotypes are known to differ in the proportion of muscular section (foot) contributing to the whole body, it remains to be checked which of the above explanations better explains this high proteome differentiation. Results from this new study suggest that tissue heterogeneity cannot explain the high proteomic differences observed between ecotypes of *L. saxatilis*. On the other hand, different estimates of proteome differentiation between ecotypes were obtained depending on the number of replicates used in each study (ranging from 7 to 30%). A reanalysis of previous published data shows a clear positive relationship between the degree of proteome differentiation observed and the number of biological replicates used in the analysis. This stresses the importance of investigating the effect of sample size on gene expression analyses. Finally, the results from the present study also discard the idea that observed proteome differences between ecotypes under similar laboratory conditions might be due to an interaction effect between common garden and the two different ecotypes. General conclusions drawn from the present study could be useful for setting up future gene expression studies in other species.

1. Introduction

Littorina saxatilis (Olivi 1972) is an ovoviviparous marine gastropod with sexual dimorphism, living in the intertidal rocky shore along the Atlantic coast. In the Galician coast (northwest of the Iberian Peninsula) two different ecotypes of this species are found adapted to different environments. These two ecotypes, known as RB (ridged and banded) and SU (smooth and unbanded), differ both at morphological and genetic levels (reviewed in Rolán-Alvarez, 2007). The SU is smaller than RB, but seems to have a more developed foot which is proportionally larger when compared to whole body, than individuals of RB ecotype (Conde-Padín et al., 2007). This could be explained by differential adaptation to distinct environmental conditions: RB individuals live in upper rocky-shore areas, where wave action is low but they must deal with stressing conditions due to the high temperatures and sun exposure (drying), low humidity, as they spend a long time out of water, and also co-inhabiting with different predators. Individuals from the SU ecotype live instead in lower rocky-shore areas under strong wave action, so they spend much of the day time submerged under conditions of high humidity and less heat stress, hence less exposed to desiccation. Individuals of this ecotype share a habitat with mussels and barnacles, and presumably an adaptation to avoid dislodging due to wave action is a wider shell aperture to accommodate a larger muscular foot to allow them to remain attached to the rocky substrate. Previous results have provided evidence of ecotype distribution being caused by differences in terms of viability and survival (Cruz et al., 2004a,b; Rolán-Alvarez et al., 1997). The morphological differences observed between ecotypes with regard to foot size and shell-aperture size were proposed to be a natural selection outcome in this species (Rolán-Alvarez, 2007). Individuals of both ecotypes meet and mate at the midshore were they coexist in sympatry and appear to be partially reproductively isolated. Current evidence points to micro-habitat preferences and mate choice based on size as the most important mechanisms contributing to this reproductive isolation (Conde-Padín et al., 2008).

Many morphological and genetic studies in this species have been developed, however there is a lack of studies at the gene expression level. The study of the proteins expressed by a genome (proteome) brings several advantages over DNA and even RNA-level studies (reviewed in

Abbreviations: 2-DE, two-dimensional electrophoresis; MS, mass spectrometry; IEF, isoelectric focusing; RB, ridged and banded; SU, smooth and unbanded; ANOVA, analysis of variance.

* Corresponding author. Tel./fax: +34 986813828.
E-mail addresses: angel.p.diz@uvigo.es (A.P. Diz), rolan@uvigo.es (E. Rolán-Alvarez).

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Diz et al., 2012a). In brief, the proteome is the result of the expression of the organism genotype and its interactions with the environment. Thus, the proteome can be regarded as the molecular phenotype and its study allows the detection of variations due to differences not only in the genotype, but also in their expression levels and posttranscriptional and posttranslational modifications, something that cannot be predicted from studies at DNA level alone. There are several techniques used in quantitative proteomics to study the proteome of an organism, cell or tissue at a given time. One of these techniques is the separation of proteins by two-dimensional electrophoresis (2-DE) according to their isoelectric point and molecular weight. Then proteins on 2-DE gels are stained, 2-DE scanned images analysed by specific software and proteome patterns analysed statistically. This technique allows for the study of variation of protein expression in biological samples under different conditions. Furthermore, proteins of interest can be identified by mass spectrometry (MS) analysis (reviewed in Diz et al., 2012a).

So far, there have been three quantitative proteomic studies in *L. saxatilis* using a 2-DE + MS approach (Diz et al., 2012b; Martínez-Fernández et al., 2008, 2010). The important common features of these studies were, 1) the quantitative proteome analyses between the two ecotypes (RB and SU), 2) the use of whole snails (unshelled), 3) the use of a pooling approach in protein sample preparation, and 4) the same geographical origin of all samples (Silleiro, Oia, Spain). In addition, in all cases the snails were maintained in common-garden conditions, from several days (1–10) to 6 months, before they were snap frozen in liquid nitrogen for further proteomic analyses. Altogether these studies provided evidence of, 1) high proteomic differences between ecotypes of up to 30% of the proteome analysed (this percentage presumably depending on the number of biological replicates used, which affects the statistical power), 2) similar changes in proteome expression levels in two ecotypes during ontogeny from embryonic to adult stages, and 3) low proteome differences between sexes (<5%). Remarkably these studies also confirmed the role of arginine kinase and fructose-1,6-bisphosphate aldolase as candidate proteins underlying molecular mechanisms of adaptive processes in these two ecotypes (but see discussion, box 2, in Diz et al., 2012a).

However, as mentioned above, such high differentiation in proteome expression between ecotypes could be caused either by distinct protein expression levels within tissue/s or by different tissue proportions within individuals in the two ecotypes. This latter explanation is related to an assumption underlying quantitative gene expression studies when the whole individual is used for the analysis, *i.e.* the homogeneity distribution of the different tissues (proportion of each tissue to the whole individual) in all individuals across groups of samples (Whitehead and Crawford, 2006). As stated above, a different foot size (muscular section) between both ecotypes was reported, which suggests the existence of possible tissue heterogeneity between individuals of both ecotypes, so a specific test is necessary to discern between both explanations. Another point is the expected difference in the gene expression profiles among different tissues within an organism. In this regard, it was recently reported that differences in protein expression profiles among tissues within an organism have quantitative rather than qualitative nature (Geiger et al., 2013; Gry et al., 2010). On the other hand, diverse estimates of proteome differentiation between ecotypes were obtained depending on the number of replicates used in each proteomic study (ranging from 7 to 30%; see Diz et al., 2012b; Martínez-Fernández et al., 2008). This suggests the need for investigating the effect of sample size on gene expression analyses not only in *L. saxatilis* but also in other species in order to assess whether or not the statistical power is high enough in a research study.

In this paper, the proteome of RB and SU ecotypes of *L. saxatilis*, from the same population analysed in previous proteomic studies, was compared either for the foot (muscular section) or for whole individual (control, as in previous studies). We tested whether high reported proteome differences between ecotypes could be better explained by true differences in expression in the same tissue/s between ecotypes or by tissue heterogeneity between ecotypes. Therefore, an equal or higher proteome differentiation between ecotypes, when the foot (muscular section) compared to the whole individual (control) is analysed, would support the former rather than the latter hypothesis. On the contrary, a significantly smaller proteome differentiation between ecotypes would suggest a more relevant role for the tissue heterogeneity hypothesis. To investigate the effect of sample size on proteome differentiation estimates, a re-analysis of published proteomic data in this same species was carried out (Diz et al., 2012b) by randomly choosing a different number of biological replicates within each ecotype. Results from this study suggest that tissue heterogeneity does not explain the high proteomic differences observed between ecotypes of *L. saxatilis*. Moreover there is an obvious and clear positive relationship between the degree of proteome differentiation observed (% of protein spots differential expressed between ecotypes) and the number of biological replicates (*i.e.*, sample size) analysed. General conclusions are drawn, which can be valid for other similar gene expression studies.

2. Materials and methods

2.1. Sampling and experimental design

L. saxatilis individuals (shell height range: 4–8 mm) from the two different ecotypes (RB and SU) were collected during the same day (April 2012) from an intertidal rocky shore area in Silleiro Cape, Oia, NW Spain (42°06′15″N; 8°53′56″W). After collection, all individuals were snap frozen in liquid N_2 in order to get a proteome snapshot of these two *L. saxatilis* ecotypes (RB and SU) from the wild. Samples were transported to a laboratory and kept frozen (−80 °C) until further analyses.

In order to test the two main hypotheses of this study, *i.e.* whether high reported proteome differences between ecotypes were caused by differences in protein expression within tissue or by tissue heterogeneity between ecotypes, the following approach was used. Firstly, once the shell was removed, the foot (muscular section) from different individuals of each ecotype was dissected under the stereoscopic microscope and extracted for further proteomic analysis. Secondly, whole unshelled individuals from different ecotypes were used as a control. Samples were labelled as "M" (Muscular section) and "T" (Total-whole individual), while "RB" or "SU" code was also added to distinguish samples from each ecotype. Despite a low sex-bias effect reported in the proteome expression analyses (see Diz et al., 2012b), only female adults were used in this study in order to control for "sex" factor.

A pooling sample strategy was followed after protein extraction, an appropriate strategy in terms of reducing the biological variation among biological replicates, hence increasing the statistical power (Diz et al., 2009; Kendziorski et al., 2005). This was successfully applied in previous studies in the same marine organism (Diz et al., 2012b; Martínez-Fernández et al., 2008, 2010) when a high inter-individual variation in gene expression patterns is expected (non-clonal organism). Pooled samples were made with 15 individuals each, either for "M; Muscle" or "T; Total" samples, and the same followed for RB and SU ecotypes. When following a pooling approach, the higher the number of individuals pooled in each sample the better and an equal contribution of each individual sample to the pool is expected (see Diz et al., 2009, 2012a). The number of individuals chosen to make each pooled sample resulted from the need to deal with the low tissue quantity obtained after dissection of muscular section (the foot), and also to substantially minimize the variation between pooled samples (biological variation). Two pooled samples (biological replicates) were prepared for each combination of "Tissue" (M and T) and Ecotype (RB and SU) samples, representing 8 biological replicates in total (but including information from 120 specimens).

In addition to this new experimental data, we used published data from Diz et al. (2012b), which consisted of 549 protein spots analysed in pooled samples of RB and SU ecotypes (n = 9 biological replicates