0014-4754/95/050539-06$1.50 + 0.20

Developmental genes as a potential tool in population ecology of complex animal life cycles?

B. Schierwater

Department of Ecology and Evolution, Fachbereich Biologie, J.W. Goethe-Universität, Siesmayerstr. 70, D-60054 Frankfurt (Germany), Fax +49 69 7982 4820; and Yale University, Department of Biology, 165 Prospect Street, New Haven (Connecticut 06511, USA)

Abstract. Many aquatic invertebrates possess complex life cycles, which include a switch from a vegetative to a sexual mode of reproduction. While sexual reproduction is usually linked to slow rates of propagation, vegetative reproduction leads to high rates of clonal propagation and a fast increase in population size. I have attempted to identify developmental genes which are differentially expressed between sexually and vegetatively reproducing individuals. Quantitative assays of the expression of those diagnostic genes could be applied to field samples to gain information on the reproductive status of the sample. Here I outline a general strategy for identifying and testing the usefulness of homeobox genes as candidates for regulatory genes whose expression patterns are indicative of a sexual or vegetative developmental phase.
Key words. Life cycle; sexual reproduction; population dynamics; homeobox genes; hydrozoa.

Complex animal life cycles

In order to extend their ecological niches, a large number of animal species from different phyla evolved 'complex' reproductive strategies. Switching the mode of reproduction within a life cycle may be used to produce different types of offspring, which are adapted to different environmental conditions[2,8,13,23]. If one and the same individual reproduces consecutively by means of two different modes of reproduction we call this an 'alternation of reproductive modes'. Examples are known from a variety of marine and freshwater taxa, including porifera, hydrozoa (e.g. *Hydra*), flatworms (e.g. *Mesostoma*), bryozoa, and crustaceans (cladocera) (e.g. refs 29, 34). More common than an alternation of reproductive modes is an 'alternation of generations', in which two or more reproductive modes are linked to different generations, i.e. one individual uses only one mode of reproduction. A primary alternation of generations (alternation between sexually produced gametes and asexually produced agametes) is 'normal' for protists and mesozoa. A secondary alternation of generations between bisexually and monosexually, i.e. parthenogenetically, reproducing generations (heterogony) is typical of freshwater rotifers. A secondary alternation of generations between a bisexual and a vegetative (most often called 'asexual', see ref. 24 for definitions) generation is found in several marine and freshwater taxa, such as in all Thaliacea, most Hydrozoa and Scyphozoa, some flatworms (Cestoda), and several annelid worms.

The wide distribution of complex life cycles among most major animal groups makes complex life cycle strategies a generally important factor in ecology. In fact, life history theory almost demands that life cycles in general should be complex, since this is believed to be the most effective means of adaptation to variable environments (e.g. refs 21, 31, 33). A bisexual mode of reproduction is normally linked to the generation of genetic diversity, and most often to the production of more robust (e.g. diapausing or overwintering) developmental stages at the expense of replicative efficiency (i.e. number of offspring produced per unit time and effort). In contrast monosexual (parthenogenetic) or asexual reproduction normally leads to high rates of propagation at the expense of genetic recombination, since clonal lineages are almost always produced. The number of offspring produced per unit time can be several orders of magnitude higher if monosexual or asexual instead of bisexual reproduction is used (e.g. refs 23, 24). These consequences for population dynamics are well known from field observations and have sparked numerous theoretical life history models. The latter aim to explain and predict population dynamics related to different reproductive strategies and changing environmental conditions[33]. However, in one sense the usefulness of theoretical models for ecologists has been rather limited. In monitoring various environmental factors along with the population parameters, they have been able to detect correlations and eventually causal relationships between these parameters and certain environmental factors only after changes in the mode of reproduction have occurred. In many instances, however, it would be highly desirable to predict changes in life history parameters beforehand, that is, before the consequences of a switch in the mode of reproduction become visible in a population. To establish causal relationships between changing environmental factors (e.g. pollution) it is of crucial importance to detect organismal responses as early as possible. The latter

may be achieved at the level of gene expression which precedes the changes of the phenotype.

If one could identify developmental genes with expression patterns which correlate with, or better precede, a certain mode of reproduction, one would be able to survey developmental and reproductive stages of individuals or populations by quantifying the expression of those genes. Thus, by means of Northern analyses or antibody studies on pooled large field samples, one could obtain quantitative estimates of the developmental and reproductive status of populations. In order to identify suitable genes one may search 1) for any genes which are differentially expressed between relevant life cycle stages (e.g. by means of differential screenings of cDNA samples from the different life cycle stages), or 2) only within regulatory genes, which in principle have the potential to be major factors in the control of developmental life history changes.

Transcription factors are among the likely candidates for regulatory genes whose expression precedes detectable phenotypic changes connected with life history changes. Homeobox genes encode transcription factors, and are known to establish fields of positional information for subsequent morphogenetic changes[1,15,16,36], such as those that often accompany alternations of reproductive modes. In the following I shall concentrate on homeobox genes and briefly outline a molecular approach which aims to identify regulatory genes that are involved in major life history changes in complex animal life cycles.

A model system: the life cycle of *Eleutheria dichotoma*

The hydroid *Eleutheria dichotoma* is, for several reasons, particularly suited as a model system to study the genetics of a complex life cycle[12,22–24]. The metagenic life cycle of *E. dichotoma* includes a vegetative polyp stage, and both sexual and vegetative medusa stages. In the natural environment, rocky eulitoral zones in the Mediterranean, the perennial polyps bud off primary medusae from spring through autumn. The medusae, which are not capable of swimming, walk on their tentacles away from the polyps and onto their substrate, the green alga *Ulva*. Young medusae undergo several cycles of vegetative production by immediately budding off secondary daughter medusae, which themselves produce another generation of secondary daughter medusae, and so forth (fig. 1).

Most medusae become sexually mature after they have produced a variable number of vegetative offspring. Sexual medusae are hermaphrodites and produce eggs and sperm simultaneously. Embryos are produced by means of selfing and develop in a broad pouch until their release as planula larvae[10]. The planulae metamorphose into sessile primary polyps, which give rise to new polyp colonies (fig. 1). All developmental states of *E. dichotoma* can easily be cultured as clonal lineages in the laboratory, and life history parameters can be varied experimentally using well established techniques.

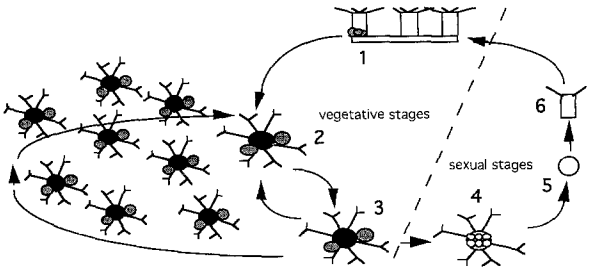

Figure 1. The metagenic life cycle of *Eleutheria dichotoma*. 1 = polyp colony; 2, 3 = vegetative medusae; 4 = sexual medusa with embryos; 5 = planula larva; 6 = primary polyp. By means of sexual reproduction it takes several weeks to produce reproductive offspring, whereas by means of vegetative reproduction, offspring production increases exponentially with mean doubling times as short as 3–4 days[23]. Further explanations are given in the text.

The molecular strategy

In order to survey candidate genes which might mark sexual versus vegetative life history traits in the medusa stage, we identified five homeobox genes (*Cnox-1* to *Cnox-5*; *Cn*idarian homeob*ox* genes *1* to *5*; ref. 27; Kuhn et al. in prep.). We are studying the expression patterns of these genes by means of Northern analysis and antibody staining of different developmental and reproductive stages of *E. dichotoma*. The overall strategy includes:

1) PCR amplification of 77 bp homeobox fragments,
2) genomic library preparation and cloning,
3) cDNA cloning by RACE and LA-PCR,
4) *Cnox* protein expression and production of polyclonal anitbodies, and
5) gene expression analysis.

The schematic drawing in figure 2 outlines the different steps that led to the characterization of the first two genes and to the production of polyclonal antibodies against the *Cnox-1* and *Cnox-2* proteins. Not shown in figure 2 are Northern analyses, which are the means to test for life cycle stage specific expression of the genes immediately following step 3). The experimental strategy may serve as a general guideline to approach any metazoan species with a complex life cycle of interest. However, the overall strategy is rather time consuming and for most applications steps 1) and 3) followed by Northern analysis may be sufficient. If step 4) is also performed, quantitative Northern analyses or Western blots, or both, can be used to quantify life cycle stage specific gene expression at the mRNA and/or protein level. In the following I will use the first two genes, *Cnox-1* and *-2*, as examples to illustrate the different steps.

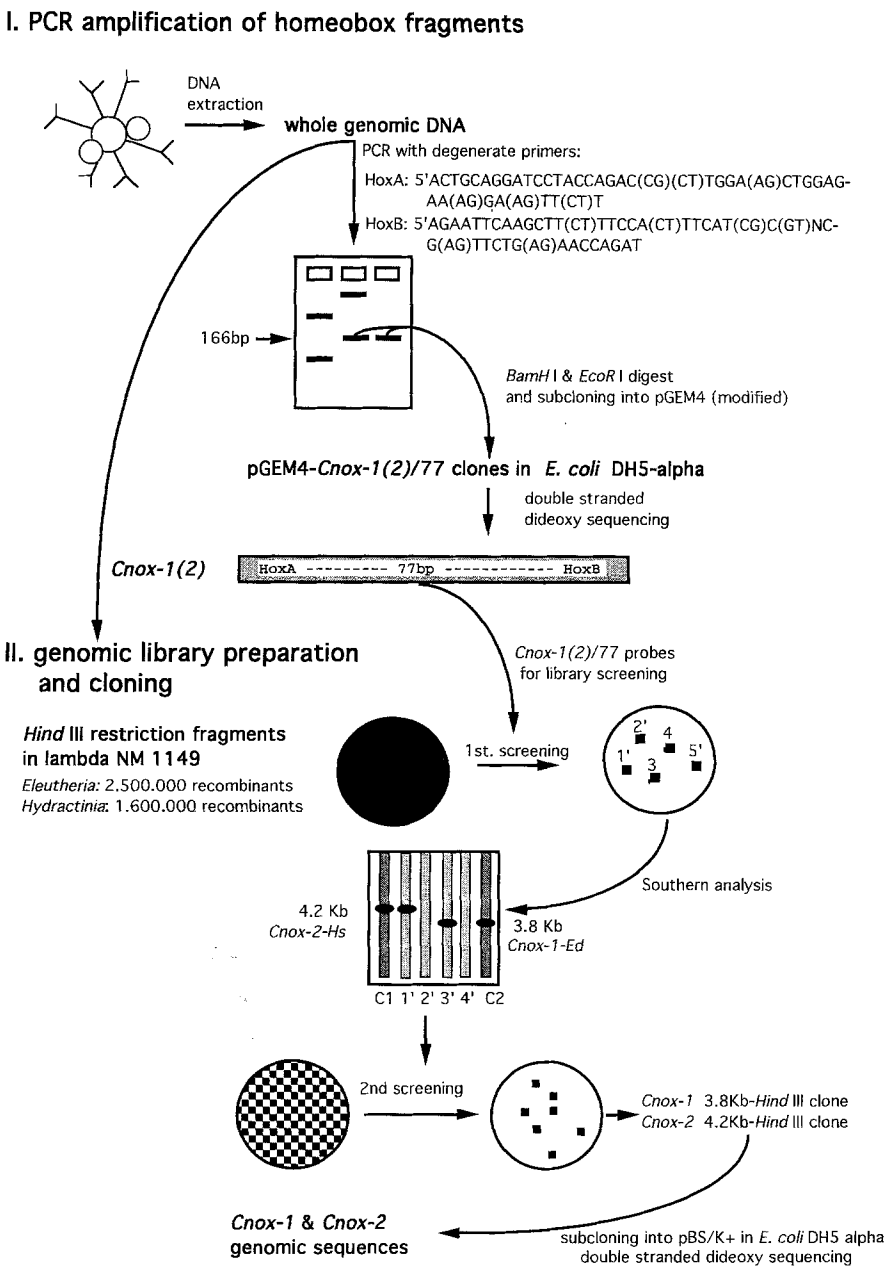

Figure 2. Molecular stragegy for detecting and cloning homeobox genes (part I and II). Explanations are given in the text.

1) PCR amplification of 77 bp homeobox fragments

Whole genomic DNA was extracted from *Eleutheria dichotoma* and subjected to PCR amplification with degenerate primers. Primer sequences correspond to conserved regions in the first and third helices of *Antennapedia* class homeodomains[14] and are expected to amplify 77 bp fragments of *Antp*-type homeoboxes[27]. Amplification products of the expected size, 166 bp including primers, were digested and cloned into a modified pGEM4 vector. Alternatively, amplification products may also be cloned directly into any PCR direct cloning vector (TA cloning). Some 60 clones were sequenced and initially two new homeobox genes found, *Cnox-1* and *Cnox-2*. Meanwhile there are several other universal primer sets available, which are known to amplify different homeobox fragments also from other classes of homeobox genes. From *Eleutheria* we have so far cloned and sequenced a total of 5 *Cnox* genes, which shows similarities to different classes (Kuhn et al., in prep.).

2) Genomic library preparation and cloning

In order to obtain full length homeobox sequences and furthermore a complete characterization of the genes (including the promotor sequences) I have prepared genomic *Hind* III libraries in phage (insertion vector lambda NM 1149). Using the strategy shown in figure 2 (part II), I have cloned 3.8 kb and 4.2 kb *Cnox-1* and *Cnox-2 Hind* III fragments which contain the full length

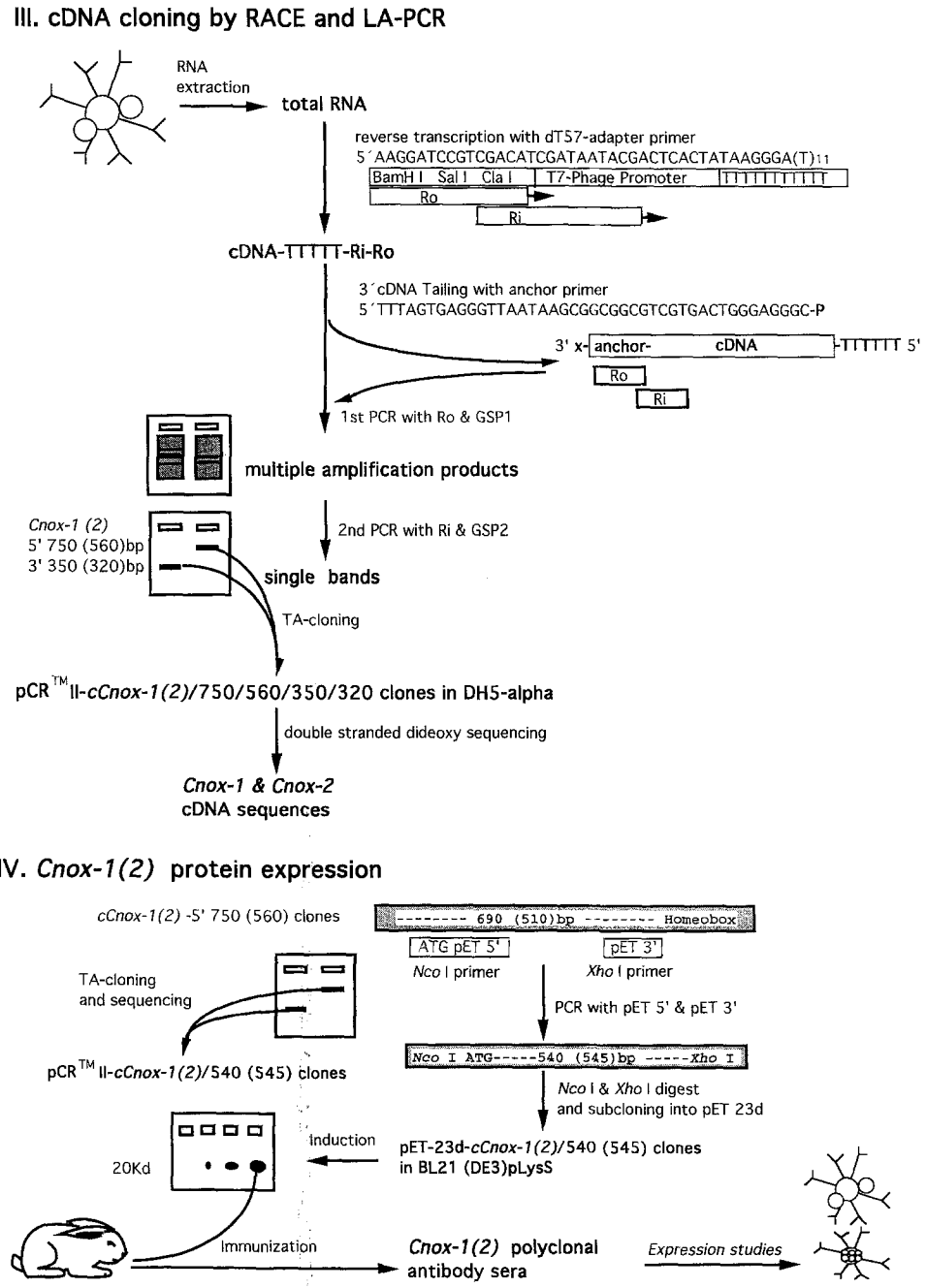

Figure 3. Molecular strategy for cDNA cloning (III) and protein expression of homeobox genes (IV). Note, the Northern analysis step, which immediately follows the cDNA cloning is not shown in the simplified scheme. Explanations are given in the text.

homeoboxes of the genes. The full length homeobox sequences provide valuable information for the design of gene specific primers for cDNA amplification by means of RACE and LA-PCR (refs 7, 35) (fig. 3, part III). However, the main reason for the genomic cloning of the genes was the complete characterization of the genes (including the identification of the promotor sequences) which will be useful in another context, e.g. to design transgenic animals. In the context of this chapter, this step can be relatively time consuming and may be omitted.

3) cDNA cloning by RACE and LA-PCR

cDNA cloning and sequencing is essential for both the generation of gene specific probes for Northern anlayses and the production of polyclonal antibodies. Two recent protocols, RACE (rapid amplification of cDNA ends; ref. 7) and LA-PCR (ligation anchored PCR; ref. 35), provide rapid means to amplify and clone 3′ and 5′ cDNA ends, if only a small internal stretch of DNA sequence is known from the gene of interest. We used nested primer designs both for RACE to clone the 3′ ends and for LA-PCR to clone the 5′ ends of *Cnox-1*

and *Cnox-2* (fig. 3, part III). The same cDNA pools were used for both protocols by using the dT57-adapter primer from Frohman and Martin[7] for the reverse transcription of mRNA. Not shown in the simplified scheme is a Southern analysis which we included after the first amplification reactions to verify the presence of amplified cDNA fragments from the genes of interest. This additional step is especially recommended, if no discrete bands get amplified in the first reaction (using the outer primers Ro and GSP1) and several bands get amplified during the second reaction (using the inner primers Ri and GSP2) (cf. ref. 28). The verified products were cloned directly into a TA cloning vector for PCR products (pCR™II Invitrogen or pGEM-T Promega) and sequenced.

Only 5′-cDNA products are shown in the scheme, since only those were used for protein expression (see below). The 5′-cDNA ends outside the homeobox of each gene could be used as templates to generate probes for Northern analysis. However, in our case the products still contained some 20–40 bp of homeobox sequence (since we designed the gene specific primers for cDNA amplification from inside the homeobox), and thus were not directly suited as probes in Northern analyses. The high sequence conservation inside the homeobox would bear the risk of cross hybridization to other homeobox genes. The same argument applies to the expression of the proteins for the production of polyclonal antibodies, if parts of the conserved homeodomain are part of the antigen.

At this point the suitability of the cloned genes as life cycle stage diagnostic markers should be tested by Northern analysis. Any cDNA fragment without homeobox sequence may be used as a probe against total or mRNA from the different life cycle stages of interest. If suitable genes have been identified, quantitative Northern analysis can be used to estimate the relative ratio of a certain life cycle stage in mixed samples. The latter must be achieved by calibrating the signal intensity of a Northern hybridization against known ratios of different life cycle stages in mixed RNA samples (cf. ref. 11).

4) *Cnox-1(2)* protein expression

If genes have been identified that are differentially expressed between the life cycle stages of interest, one may produce polyclonal antibodies against the corresponding proteins. This way, quantitative antibody assays may complement or replace quantitative Northern analysis. Antibody assays are easier and faster to perform and the preservation of proteins from field samples is less problematic than the preservation of RNA.

The 5′ coding region of homeobox genes is normally significantly longer than the 3′ region, and thus offers more potential antigenic epitopes. The homeodomain itself should not be part of the expressed protein if this is used as an antigen to produce polyclonal antibodies. For *Cnox-1* and *Cnox-2* we used the 5′ cDNA ends outside the homeobox for cloning into the expression vector (fig. 3, part IV). From a variety of expression vectors available we chose pET 23d (Novagene) for protein expression, which gave us the highest levels of protein expression. In general, protein expression and production of polyclonal antibodies can be achieved by standard protocols[20].

5) Gene expression analysis

Western blot and whole-mount immunocytochemistry studies on different sexual vegetative medusa stages are in progress in order to detect and quantify *Cnox-1*, and *-2* gene expression in the life cycle of *E. dichotoma*. Using affinity purified polyclonal antibodies, preliminary data suggest differential gene expression for *Cnox-1* in different medusa stages (Schierwater et al., in prep.).

Other systems

The outlined strategy for the identification and preparation of homeobox genes for Northern analysis or antibody studies uses standard protocols and thus can be easily adopted to other study organisms. Although the final step of applying expression tests to field studies has not been done for any study organism yet, it seems likely that this will only be a matter of short time. Once the suited cDNA clones for making the probes for Northern analysis have been made available, the success of the overall approach will depend on finding differential expression patterns of the genes between the different reproductive modes of interest. Apparently, chances for success will increase directly with the degree of non-overlapping developmental processes between different life history stages in the species under investigation.

In the case of *Eleutheria* I expect little difficulties, since here the developmental processes accompanying sexual versus vegetative reproduction are highly exclusive. Vegetative medusae are characterized by vegetative growth developing the medusoid form, which requires little cell fate determination (except limited I-cell differentiation). In contrast, sexual medusae do not feed and grow and developmental processes are almost exclusively related to cell fate determination for gametogenesis and early embryogenesis (cf. refs. 4, 5, 6, 9, 17–19, 36). A similar situation may be expected for other species with complex life cycles. If, however, the developmental differences between the life history traits (reproductive modes) is less exclusive, one has to expect a greater chance that certain homeobox genes may be expressed in both traits. Also, specific homeobox genes may be expressed in different tissues at different times in ontogeny. The latter could require that more genes need

to be screened before finding the suitable one(s). In these cases it would not be feasible to apply the whole strategy outlined above, rather than doing only step 1) and 3), i.e. the identification and cDNA cloning of the genes in order to verify the suitability of the identified genes by Northern analysis. Since the number of homeoboxes and homeobox genes identified by molecular geneticists and developmental biologists for other reasons in a variety of species is steadily and rapidly increasing, some future applications may require as little as part 3) of the outlined strategy (i.e. the homeobox lacking cDNA cloning). If, however, homeobox genes are not yet readily available for a species of interest, it might be faster to screen for any diagnostic developmental gene, that is to replace step 1) by differential screenings of cDNA samples from relevant life cycle stages. By means of Northern analysis diagnostic genes may be found, which can elegantly be used by ecologists as a new tool to assay reproductive traits and thus predict population dynamics in animal species with complex life cycles.

Acknowledgements. I am grateful to John McCarthy (GBF Stöckheim) and Leo W. Buss (Yale University) for collaborative research on genomic cloning and expression studies. I thank Bruno Streit and Kerstin Kuhn, and I acknowledge financial support from BMFT (Helmholtz-Programm), DFG (Schi 227-/3-1) and NATO (CRG-910094).

1 Averoff, M., and Akam, M., HOM/Hox genes of artemia: implications for the origin of the insect and crustacean body plans. Curr. Biol. *3* (1993) 73–78.
2 Calow, P., Asexual reproduction in protozoa and invertebrates. J. theor. Biol. *96* (1982) 401–424.
3 Chomczynski, P., and Sacchi, N., Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analyt. Biochem. *162* (1987) 156–159.
4 Crompton, M. R., Bartlett, T. J., MacGregor, A. D., Manfioletti, G., Buratti, E., Giancotti, V., and Goodwin, G. H., Identification of a novel vertebrate homeobox gene expressed in haematopoietic cells. Nucleic Acids Res. *20* (1992) 5661–5667.
5 Duboule, D., and Dollé, P., The structural and functional organization of the murine Hox gene family resembles that of Drosophila homeotic genes. EMBO J. *8* (1989) 1498–1505.
6 Fainsod, A., and Gruenbaum, Y., A chicken homeobox gene with developmentally regulated expression. FEBS Lett. *250* (1989) 381–385.
7 Frohman, M. A., and Martin, G. R., Rapid amplification of cDNA ends using nested primers. Technique *1* (1989) 165–170.
8 Giese, A. C., and Pearse, J. S., Reproduction of Marine Invertebrates. Academic Press, New York-London 1959.
9 Graham, A., Papalopulu, N., and Krumlauf, R., The murine and Drosophila homeobox gene complexes have common features of organization and expression. Cell *57* (1989) 367–378.
10 Hadrys, H., Schierwater, B., and Mrowka, W., The feeding behaviour of a semi-sessile hydromedusa and how it is affected by the mode of reproduction. Anim. Behav. *40* (1990) 935–944.
11 Hadrys, H., Balick, M., and Schierwater, B., Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Molec. Ecol. *1* (1992) 55–63.
12 Hauenschild, C., Experimentelle Untersuchungen über die Entstehung asexueller Klone bei der Hydromeduse *Eleutheria dichotoma*. Z. Naturf. *11b* (1956) 394–402.
13 Hauenschild, C., Fortpflanzung und Sexualität der Tiere. BI Taschenbuchverlag, Mannheim-Leipzig-Wien-Zürich 1993.
14 McGinnis, W., Levine, M. S., Hafen, E., Kuroiwa, A., and Gehring, W. J., A conserved DNA sequence in homeotic genes of the Drosophila Antennapedia and bithorax complexes. Nature *308* (1984) 428–433.
15 McGinnis, W., and Krumlauf, R., Homeobox genes and axial patterning. Cell *68* (1992) 283–302.
16 Slack, J. M. W., Holland, P. W. H., and Graham, C. F., The zootype and the phylotypic stage. Nature *361* (1993) 490–492.
17 Nardelli-Haefliger, D., and Shankland, M., Lox2, a putative leech segment identity gene, is expressed in the same segmental domain in different stem lineages. Development *116* (1992) 697–710.
18 Nardelli-Haefliger, D., and Shankland, M., Lox10, a member of the NK-2 homeobox gene class, is expressed in a segmental pattern in the endoderm and in the cephalic nervous system of the leech Helobdella. Development *118* (1992) 877–892.
19 Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B., and Goodman, C. S., Expression of engrailed proteins in arthropods, annelids and chordates. Cell *58* (1989) 955–968.
20 Sambrook, J., Fritsch, E. F., and Maniatis, T., Molecular cloning – a laboratory manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour 1989.
21 Sauer, K. P., The evolution of reproductive strategies as an adaptation to fluctuating environments. Adv. Inv. Reprod. *3* (1984) 317–326.
22 Schierwater, B., Allometric changes during growth and reproduction in *Eleutheria dichotoma* (Hydrozoa, Athecata) and the problem of estimating body size in a microscopic animal. J. Morph. *200* (1989) 255–267.
23 Schierwater, B., and Hauenschild, C., A photoperiod determined life-cycle in an oligochaete worm. Biol. Bull. *178* (1990) 111–117.
24 Schierwater, B., and Hauenschild, C., The position and consequences of a vegetative mode of reproduction in the life-cycle of a hydromedusa and an oligochaete worm. Adv. Inv. Reprod. *5* (1990) 37–42.
25 Schierwater, B., Piekos, B., and Buss, L. W., Hydroid stolonal contractions mediated by contractile vacuoles. J. expl Biol. *162* (1992) 1–21.
26 Schierwater, B., Ecophysiological consequences of the occurrence of contractile vacuoles in the life cycle of the hydromedusa *Eleutheria dichotoma*. Verh. dt. zool. Ges. *85* (1992) 38.
27 Schierwater, B., Murtha, M., Dick, M., Ruddle F. H., and Buss, L. W., Homeoboxes in Cnidarians. J. expl Zool. *260* (1991) 413–416.
28 Schierwater, B., and Ender, A., Different thermostable DNA polymerases may amplify different amplification patterns. Nucleic Acids Res. *21* (1993) 4647–4648.
29 Schierwater, B., Ender, A., Schwenk, K., Spaak, P., and Streit, B., The evolutionary ecology of Daphnia, in: Molecular Approaches to Ecology and Evolution, pp. 495–508. Eds B. Schierwater, B. Streit, G. P. Wagner and R. DeSalle. Birkhäuser, Basel-Boston 1994.
30 Schummer, M., Scheurlen, I., Schaller, C., and Galliot, B., HOM/HOX homeobox genes are present in hydra (Chlorohydra virdissima) and are differentially expressed during regeneration. EMBO J. *11* (1992) 1815–1823.
31 Schmidt, B. R., Are Hybridogenetic frogs cyclical parthenogens? Trends Ecol. Evol. *8* (1993) 271–273.
32 Shenk, M. A., Bode, H. R., and Steele, R. E., Expression of Cnox-2, a HOM/HOX homeobox gene in hydra, is correlated with axial pattern formation. Development *117* (1993) 657–667.
33 Stearns, S. C., Life-history tactics: a review of the ideas. Q. Rev. Biol. *51* (1976) 3–47.
34 Streit, B., Städler, T., Schwenk, K., Ender, A., Kuhn, K., and Schierwater, B., Natural hybridization in freshwater animals. Ecological implications and molecular tools. Naturwissenschaften *81* (1994) 65–73.
35 Troutt, A. B., McHeyzer-Williams, M. G., Pulendran, B., and Nossal, G. J. V., Ligation anchored PCR: A simple amplification technique with single-sited specificity. Proc. natl Acad. Sci. USA *89* (1992) 9823–9825.
36 Wedeen, C. J., and Weislat, D. A., Segmental expression of an engrailed-class gene during early development and neurogenesis in an annelid. Development *113* (1991) 805–814.