

4

The Genus *Antirrhinum* (Snapdragon) A Flowering Plant Model for Evolution and Development

Andrew Hudson, Joanna Critchley, and Yvette Erasmus

University of Edinburgh, Institute of Molecular Plant Sciences, Edinburgh EH9 3JH, United Kingdom

ABSTRACT

The *Antirrhinum* species group comprises approximately 20 morphologically diverse members that are able to form fertile hybrids. It includes the cultivated snapdragon *Antirrhinum majus*, which has been used as a model for biochemical and developmental genetics for more than 75 years. The research infrastructure for *A. majus*, together with the interfertility of the species group, allows *Antirrhinum* to be used to examine the genetic basis for plant diversity.

PROTOCOLS

- 1 Cultivating *Antirrhinum*, 111
- 2 Propagating *Antirrhinum*, 114

BACKGROUND INFORMATION

The garden snapdragon *A. majus* has several centuries' history of cultivation as a flowering ornamental. It emerged as a model organism during early studies of inheritance and mutation (e.g., Darwin 1868) because of its diploid inheritance, ease of cultivation, and variation in morphology and flower color. Laboratory lines of *A. majus* were produced from cultivars, and a substantial collection of mutants had amassed during the course of the 20th century. This collection included lines with unstable mutations in pigment genes, which produced variegated flowers (Fig. 1a).

Transposons responsible for flower variegation were identified in the 1980s at the John Innes Centre in Norwich, United Kingdom and the Max-Planck-Institut in Cologne, Germany, allowing genes involved in flower and leaf development and in pigmentation to be isolated by transposon tagging. *A. majus* subsequently provided the first insights into the regulation of many developmental processes that are conserved in flowering plants, including the specification of flower and floral organ identity, leaf and flower asymmetry, and the pollen component of gametophytic self-incompatibility. Because *A. majus* diverged from the more commonly used eudicot model *Arabidopsis thaliana* early in the history of flowering plants, it has proven to be useful in comparative developmental studies.

The *Antirrhinum* species group also has a history of use in studies of natural variation. The close relatives of *A. majus* form a monophyletic group of about 20 species native to the Mediterranean region, particularly southwestern Europe and northern Africa. The species vary

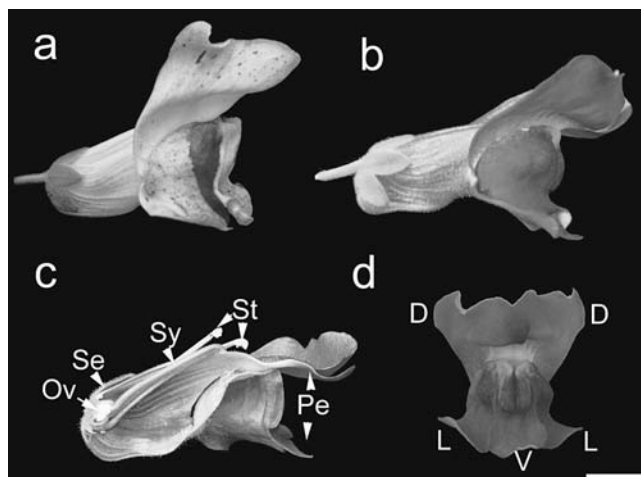


FIGURE 1. *Antirrhinum majus*. (a) The variegated flower caused by a transposon-induced mutation in a gene required for synthesis of anthocyanin pigments. Excision of the transposon can give rise to clones of wild-type cells able to produce magenta anthocyanins. (b) Wild-type *A. majus* flower. (c) Section of an *A. majus* flower, showing the four types of organs. (Ov) Ovary; (Se) sepal; (Sy) style; (St) stamen; (Pe) petal. (d) Dorsoventral asymmetry of the *Antirrhinum* corolla. (D) dorsal, (L) lateral, and (V) ventral petals. Bar, 10 mm.

widely in morphology and ecology and are adapted to different—often extreme—habitats. However, all are able to form fertile hybrids with one another and with *A. majus*, allowing the identification of genes that underlie their differences (see, e.g., Langlade et al. 2005). Population genetic studies that have been applied to *Antirrhinum* species show different population sizes, geographic distributions, and breeding systems, from self-fertility to obligate out-crossing (see, e.g., Jiménez et al. 2002; Mateu-Andres and de Paco 2006).

SOURCES AND HUSBANDRY

Seeds from a large collection of *A. majus* mutants, their wild-type progenitors (Fig. 1b), and a limited number of other *Antirrhinum* species can be obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany (<http://gbis.ipk-gatersleben.de>). Protocols for *Antirrhinum* husbandry can be found in most gardening books, and the necessary materials are readily available. Although tolerant of both frost and high temperatures, *Antirrhinum* species grow best at daytime temperatures of 17–25°C. In temperate regions, they are usually treated as half-hardy annuals and are transferred from a glasshouse to the open in the spring or are grown entirely in a glasshouse. All species grow well from seeds, most flowering within 3–4 months of sowing, and are readily propagated clonally from cuttings. Detailed methods for *Antirrhinum* culture and propagation are provided in Protocols 1 and 2.

RELATIVES OF *A. MAJUS*

Antirrhinum is a member of the asterid clade of flowering plants. The more commonly used model species *Arabidopsis* is a member of the second major clade of broad-leaved plants—the rosids—from which asterids diverged an estimated 120 million years ago. Within the asterids, *Antirrhinum* belongs to the order Lamiales, a close relative of the order Solanales, which includes other model species such as petunia and tomato. *Antirrhinum* was recently placed in the family Plantaginaceae (synonymous with Veronicaceae) following a revision of the classical family Scrophulariaceae based on DNA sequence variation (Olmstead et al. 2001).

Other aspects of *Antirrhinum* taxonomy remain controversial. The generic epithet *Antirrhinum* is now usually reserved for the monophyletic group of Old World perennials with a diploid chromosome number of 16. However, it is still applied to a broader monophyletic group that includes species with different chromosome numbers, such as the New World *Sairocarpus* and the annual *Misopates*, with which *Antirrhinum* species are unable to form fertile hybrids (see, e.g., Oyama and Baum 2004). Within *Antirrhinum sensu stricto*, a variable number of different species have been proposed and relationships between taxa are currently unresolved. These taxonomic problems largely reflect the young age of the genus (<5 million years; Gübitz et al. 2003) and the effects of hybridization (see, e.g., Whibley et al. 2006); thus, attempts to reconstruct phylogenies based on nuclear or chloroplast DNA sequence variation have so far been unsuccessful (see, e.g., Jiménez et al. 2005). In the absence of a taxonomic revision based on a resolved phylogeny, the descriptions of approximately 20 species and their likely hybrids in *Flora Europaea* (Webb 1972) provide a realistic working guide.

The genus has traditionally been divided into three subsections or morphological groups (Rothmaler 1956) that have received support from studies of isozyme and DNA variation. The subsection *Antirrhinum* includes the close relatives of *A. majus* and consists of species with similar upright growth, large organs, and pink or yellow flowers (Fig. 2a). These tend to be geographically widespread and grow in a variety of habitats. *A. majus* was probably domesticated in northeastern Spain or southwestern France from *A. pseudomajus* (also known as *A. majus* subspecies *pseudomajus*), from which it differs by having more darkly pigmented flowers, although traits such as flower color variation might have been introduced by introgression from other species. Members of subsection *Kickxiella*, in contrast, are usually restricted to rock faces and walls. They are also smaller,

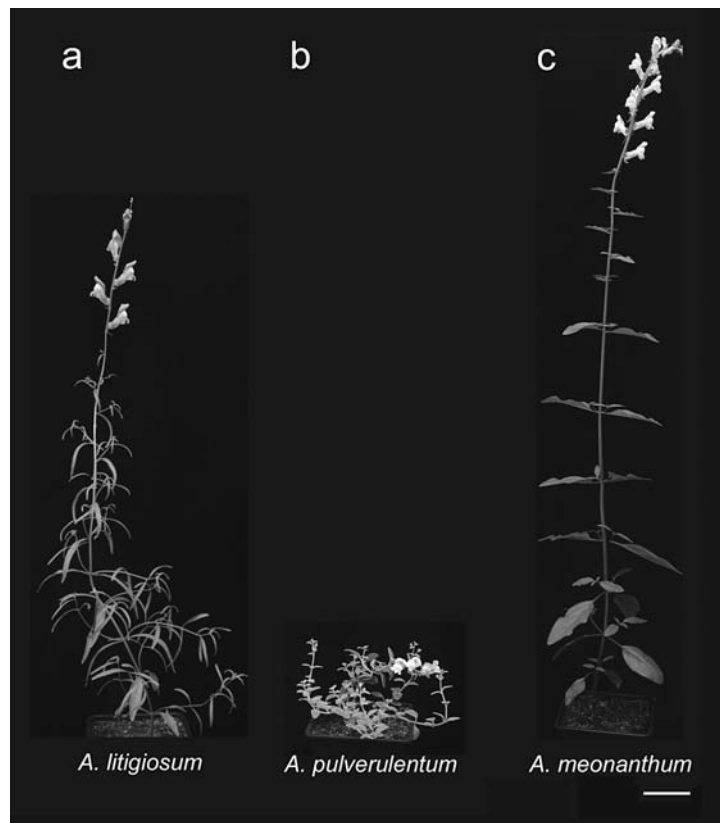


FIGURE 2. Representatives of the three traditional subsections of the genus *Antirrhinum*. (a) *A. litigiosum* (also known as *A. majus* subspecies *litigiosum*), a member of subsection *Antirrhinum*; (b) *A. pulverulentum*, a member of subsection *Kickxiella*; (c) *A. meoanthum*, a member of subsection *Streptosepalum*. Bar, 50 mm.

typically prostrate in habit, and have small white or pale pink flowers (Fig. 2b). They are geographically more restricted and many are endemic to particular mountain regions. The two members of subsection *Streptosepalum* are pale- or yellow-flowered, of upright habit, and grow in hedges and rocky outcrops in northern Spain and Portugal (Fig. 2c). Distribution maps for all *Antirrhinum* species were produced by Rothmaler (1956) and can also be found in Stubbe (1966).

USES OF THE *A. MAJUS* MODEL SYSTEM

Biochemistry

Two aspects of *Antirrhinum* biochemistry relevant to their attraction of pollinating bees have been studied in detail. First, genes encoding the enzymes involved in the production of floral scents—phenylpropanoids and isoprenoids—have been identified from *A. majus* and used to study the regulation of scent production and the effects of variation in scent composition on pollinator attraction (see, e.g., Wright et al. 2005). Second, the basis for flower color variation in *Antirrhinum* has a long history of study, including the pioneering biochemical genetics of Muriel Wheldale in the early 20th century. Structural genes encoding most enzymes involved in the biosynthesis of magenta anthocyanins and the structurally related yellow aurones have been identified and isolated through a combination of genetics and biochemistry. Several regulatory genes encoding MYB or basic helix-loop-helix (bHLH) transcription factors are known to affect the intensity or pattern of pigmentation (Schwinn et al. 2006). In parallel, many of the genes underlying natural variation in *Antirrhinum* flower pigmentation were mapped or shown to be allelic to loci identified from mutations in *A. majus*. Three of the loci involved in natural variation have been isolated: Two encode the MYB transcription factors ROSEA and VENOSA and the third is the structural gene *INCOLORATA*. Two additional genes—*SULFUREA* (*SULF*), which controls aurone pigmentation, and *ELUTA* (*EL*), which is responsible for variation in the pigment pattern within the flower—have been mapped but not yet isolated. Flower color variation in *Antirrhinum* has been shown to affect pollinator behavior, and selection at loci including *SULF* and *EL* is likely to maintain the distinction between yellow-flowered *A. striatum* and magenta-flowered *A. pseudomajus* in the face of hybridization (Whibley et al. 2006).

Development

The use of *A. majus* in parallel with other species, notably *Arabidopsis*, has led to an understanding of how the identity of flowers and floral organs is specified (for recent review, see Davies et al. 2006). These processes are broadly conserved between the asterid *Antirrhinum* and the rosid *Arabidopsis*, which have similar inflorescence and floral structures (see Fig. 1c,d for the structure of the *Antirrhinum* flower). Both species, for example, contributed to the “ABC” model of floral organ specification, in which the combination of genes expressed in each of the whorls determines the identity of the floral organs (A genes specify sepals, A + B genes specify petals, B + C genes specify stamens, and C genes specify the carpel). However, the comparative use of *A. majus* further revealed subtle evolutionary differences in the way that the development of similar flowers can be regulated. *Antirrhinum* and *Arabidopsis*, for instance, use different genes to exclude the expression of C genes from the outermost parts of the developing flower—a role originally attributed to A-function genes—leading to a reevaluation of the A-function genes in both species (Keck et al. 2003; Davies et al. 2006). Similarly, the ancestral C function appears to have been transferred to different duplicated genes after the divergence of *Antirrhinum* from *Arabidopsis* (Causier et al. 2005).

One notable aspect of the *Antirrhinum* flower that differs from *Arabidopsis* is its marked dorsoventral asymmetry (zygomorphy). Zygomorphy, considered to have coevolved with insect pollination, is apparent in the different morphologies of the dorsal, lateral, and ventral petals of *Antirrhinum* (Fig. 1d) and in stamen development. The dorsal and lateral petals are specified by the paralogous TCP transcription factor genes *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*),

which act partly by activating dorsal expression of *RADIALIS* (*RAD*). *RAD* is a protein with a single MYB transcription factor repeat that is thought to compete with the two-repeat MYB protein encoded by the *DIVARICATA* (*DIV*) gene to antagonize its ventralizing effect (for review, see Almeida and Galego 2005). *CYC*-like genes have also been implicated in the evolution of floral asymmetry in other lineages. For example, ectopic *CYC* expression has been proposed to account for the evolution of the derived radially symmetrical flower of *Mohavea*, which is within the tribe Antirrhineae (Hileman et al. 2003). *CYC*-like genes are expressed asymmetrically in both rosids and asterids, including species that have radially symmetrical flowers (for review, see Cubas 2004). This indicates that ancestral, asymmetrically expressed *CYC*-like genes might have been recruited independently to produce zygomorphic flowers in different flowering plant lineages, a view recently supported by the effects of *cyc*-like mutants in the asterid legume *Lotus* (Feng et al. 2006).

Aided by a large collection of leaf-shape mutants, *A. majus* has also been useful in studies of leaf development. These studies have included the identification of genes involved in specifying leaf identity and promoting leaf growth (see, e.g., Golz et al. 2004) and in coordinating growth in the leaf blade to ensure the development of a flat organ (see, e.g., Nath et al. 2003).

Ecology and Population Genetics

Antirrhinum species have been the subject of population genetic studies, including those aimed at assessing genetic diversity in order to inform conservation strategies for rare endemic species (see, e.g., Mateu-Andres 2004). As with other taxa, genetic diversity has generally been found to be lower in smaller populations, and the distribution of genetic variants within and between populations has been correlated with their level of self-incompatibility. In addition, several genetically well-characterized aspects of *Antirrhinum* development and physiology are relevant to their reproductive ecology, including petal cell morphology, genetic self-incompatibility, flower color, and scent production.

Whereas cultivated *A. majus* and some wild species (e.g., *A. valentinum*, *A. subbaeticum*, and *A. siculum*) are self-fertile, the majority of *Antirrhinum* species show gametophytic self-incompatibility that is determined by a single, complex *S* locus. Individuals of self-incompatible species reject pollen carrying an *S* allele that corresponds to one of their own alleles and are therefore obligate outbreeders. Through studies of relatives in the family Solanaceae, rejection of pollen was known to involve an *S*-encoded RNase that was expressed in the pistil (McClure et al. 1989), although the pollen-expressed component had remained elusive. Mapping and sequence analysis of the active *S* locus of *A. hispanicum*, followed by expression and functional studies, identified the likely pollen component to be an F-box protein involved in targeting the RNase for degradation (Lai et al. 2002).

Cells of the petal epidermis of *Antirrhinum*, like those of many flowering plants, have a conical shape that is dependent on activity of the MYB transcription factor encoded by *MIXTA*. These conical cells intensify flower color by reducing reflection, as revealed by *MIXTA* mutants with flat epidermal cells, and are important in attracting pollinating bees (Noda et al. 1994). In addition to having a role in the reproductive ecology of *Antirrhinum*, the misexpression of *MIXTA* or related genes can give rise to the formation of epidermal hairs (trichomes), indicating a common regulation of these two cell types and a role for other *MIXTA*-like genes in regulating multicellular trichome development in *Antirrhinum*. This latter function of *MIXTA*-like genes does not appear to be conserved in *Arabidopsis*, which has unicellular trichomes (for review, see Serna and Martin 2006).

GENETICS, GENOMICS, AND ASSOCIATED RESOURCES

A. majus is amenable to classical genetics. It has a relatively short generation time of about 4 months, is diploid ($2n = 16$), and is easily self- and cross-pollinated (see Protocol 2). A collection of classical mutants and corresponding wild-type lines originating from the work of Erwin Baur, Hans Stubbe, and their colleagues is maintained at the IPK. The phenotypes of most of the

mutants in this collection have been described by Stubbe (1966) and catalogued by Hammer et al. (1990). Additional mutants and wild-type lines, generated at the John Innes Centre, are described at <http://www.jic.ac.uk/STAFF/enrico-coen/Rosemary/start.htm>.

A molecular recombination map, currently comprising more than 250 loci with an average distance between loci of approximately 2 cM, has been produced for *Antirrhinum* using hybrids between *A. majus* and wild species, and the map is maintained at <http://www.antirrhinum.net>. It has been aligned with a map of classical mutants and with the eight *Antirrhinum* chromosomes using fluorescence in situ hybridization (FISH; Zhang et al. 2005). Maps constructed using hybrids between different *Antirrhinum* species are largely colinear, suggesting an absence of extensive chromosomal rearrangements, although distorted transmission of some genomic regions in interspecies hybrids can hinder genetic mapping (Schwarz-Sommer et al. 2003). Recombinant inbred lines (RILs) and near-isogenic lines (NILs) have been produced from hybrids between different *Antirrhinum* species, allowing those genes that underlie differences between species to be identified.

Native *Antirrhinum* transposons have provided the basis for a number of genetic resources. Most spontaneous mutations in *A. majus* have been found to carry transposons belonging to one of two families: the *Tam3* family, which is similar to *Ac* in maize, and the *CACTA* family, which is homologous to *Spm/En* in maize. Mutagenic retrotransposons and miniature inverted repeat transposable elements (MITEs) have also been found. Transposition of the *CACTA* and *Tam3* families occurs by excision from the donor site and reintegration elsewhere in the genome and is promoted by low temperature. A number of *A. majus* lines were selected for high transposon activity based on flower variegation and have been used for forward genetic screens and for inactivating known genes to study their functions (see, e.g., Keck et al. 2003).

Transposon excision from pigment genes, which can be controlled with temperature, has been used to mark cells for fate and growth analysis (e.g., Rolland-Lagan et al. 2003), and excision from developmental genes has been used to examine the cell autonomy of gene action (see, e.g., Golz et al. 2004). In many cases, it has been possible to establish stable periclinal chimeras in which layers of the shoot apical meristem comprise genetically different cells as the result of transposon excision (see, e.g., Carpenter and Coen 1995). Such chimeras can be maintained by vegetative propagation through cuttings.

Although stable transgenic *Antirrhinum* can be produced using *Agrobacterium tumefaciens* (Cui et al. 2003), transformation efficiency is low. Particle bombardment and *Agrobacterium* infiltration of developing petals have been used successfully to obtain transient expression of pigment genes and suppression by RNA interference (RNAi; Schwinn et al. 2006; Shang et al. 2007).

Bacterial artificial chromosome (BAC) libraries have been produced from *A. majus* (Causier et al. 2005) and from an *A. majus* × *A. hispanicum* hybrid (Lai et al. 2002). Although the genome size of *A. majus* has been estimated at 430 Mb (Bennett and Leitch 1995), the recovery of clones from genomic libraries indicates that it might be at least twofold larger; therefore, the depth of coverage of existing BAC libraries is uncertain. Currently, only short BAC contigs have been assembled to allow positional cloning in specific target regions (Cartolano et al. 2007). FISH has been used to locate BAC clones on *Antirrhinum* chromosomes (Zhang et al. 2005; Yang et al. 2007). An expressed sequence tag (EST) collection of approximately 12,000 unigenes from *A. majus* is available for searching at <http://www.antirrhinum.net>. Other DNA libraries include cDNA clones in yeast one-hybrid and two-hybrid vectors (see, e.g., Egea-Cortines et al. 1999).

TECHNICAL APPROACHES

Antirrhinum species are amenable to molecular genetic techniques. Most protocols that have been developed for *Arabidopsis*, including those for nucleic acid, protein, and organelle purification and for detection of RNA and proteins in situ, can be applied directly to *Antirrhinum*. Here, we present protocols for cultivating (Protocol 1) and propagating (Protocol 2) *Antirrhinum* plants.

Protocol 1

Cultivating *Antirrhinum*

In this protocol, we describe methods for cultivating *Antirrhinum* species. These plants are easily grown, provided that they have sufficient light and are not overwatered. In good conditions, most species will flower and produce seeds within 3–4 months. Strongly growing plants should suffer from few pests or diseases, but we also prescribe methods for handling microbes and insects that commonly damage *Antirrhinum*.

MATERIALS

The recipe for the item marked with <R> is on page 117.

Reagents

Agar (0.1%, w/v) (optional; see Step 1)

Compost <R>

As an alternative to the peat-based compost recipe provided, Antirrhinum can be grown in loam-based compost (e.g., John Innes No. 1) with additional feeding as required. Antirrhinum will grow well in a range of soils and commercial composts, but avoid those containing ammonium-based fertilizers, which encourage fungal wilting diseases.

Gibberellin (10 μ M) (optional; see Step 3)

Seeds

Seeds from a large collection of A. majus mutants and from a limited number of other Antirrhinum species can be obtained from the IPK (<http://gbis.ipk-gatersleben.de>). Other seeds are available from members of the Antirrhinum research community.

Equipment

Canes and wire ties (see Step 9)

Capillary matting (optional; see Step 7)

Covers for pots, clear plastic

Glasshouse (17–23°C, <70% humidity), with adequate ventilation and fans (see Step 8) and supplemental lighting in winter (see Step 5)

Pots (5 cm)

Larger (e.g., 10 cm) pots may also be needed (see Step 7).

METHOD

1. Fill each 5-cm pot with lightly firmed compost. In each pot, sow multiple seeds on the surface of the compost.

Alternatively, seeds can be sown singly in 5-cm pots, which eliminates the need to transplant the seedlings in Step 4. To facilitate sowing, the seeds can be suspended in 0.1% agar and pipetted onto the soil.

2. Cover the pots with a clear plastic cover to keep the soil moist.

3. Keep the plants at approximately 17°C.
A. majus laboratory strains and cultivars will germinate within 7–10 days, whereas seeds of wild accessions may show dormancy. Germination can be encouraged by soaking seeds in 10 μM gibberellin solution at 4°C for 3–5 days before sowing.
4. After germination, transplant the seedlings individually to 5-cm pots for flowering in the glasshouse.
Alternatively, transplant the plants to soil outside, once the weather is warm enough.
5. Keep plants under high light ($>150 \mu\text{M m}^{-2}\text{s}^{-1}$).
Flowering is promoted by long days; therefore, strong supplemental lighting is usually needed in glasshouses during the winter to provide a long day (~16 hours of light) and sufficient intensity for growth.
6. Maintain the plants at a temperature of 17–23°C.
A nighttime drop in temperature to 15–17°C increases apical meristem size and encourages robust stem growth.
7. Water the plants as necessary.
*Compost should not be allowed to dry to the point where plants wilt, and during the summer, it may be necessary to water the plants twice daily in 5-cm pots. Increasing the size of the pot (e.g., to 10 cm) and placing pots on capillary matting can reduce the need for watering. Avoid wetting the foliage when watering to discourage fungal infections. All *Antirrhinum* species are intolerant of waterlogged soil. Avoid standing pots in water.*
8. Use adequate ventilation and fans, if necessary, to maintain the relative humidity below 70%.
9. Using canes and wire ties, stake plants as necessary.
*A. majus grown under high light and in lower temperatures may not require support. Several species in the subsection *Kickxiella* are naturally procumbent and can be allowed to trail over the sides of the pot.*
10. Monitor the plants for pests and disease.
Strongly growing plants should suffer from few pests or diseases, but if they do, see Troubleshooting.

TROUBLESHOOTING

Problem (Step 10): Plants are wilted.

Solution: Wilting can be caused by either infection with *Pythium* species or lack of water. If the compost is moist, the wilting is caused by *Pythium*. In this case, drench the compost with a solution of commercial fosetylaluminium fungicide (e.g. Aliette from Bayer CropScience), following manufacturer instructions. Alternatively, if wilting is due to drought, water the compost well or soak it by submerging the pot in water for several minutes. The shoots of plants that have wilted due to mild *Pythium* infection or short-term drought should recover. If not, cut them off and allow new shoots to grow.

Problem (Step 10): Plants are infected with other fungal and oomycete diseases (mildews, *Botrytis*, and rust). (Mildews are apparent as grey or white powdery growths on leaves; *Botrytis* causes rapid localized death of shoots, leaves, and flowers, with dead tissues appearing dry and pale brown. Rust is first apparent on stems and on the undersides of leaves as lighter green circles that subsequently erupt into brown, spore-bearing pustules.)

Solution: Spray the shoots with suspended sulphur or other proprietary fungicide, using manufacturer recommendations. Remove dead and/or badly infected tissues.

Problem (Step 10): Shoots and flower buds show small lesions of dead tissue, and pollen is missing from anthers.

Solution: Plants may be infested with the western flower thrip *Frankliniella occidentalis*. Adult thrips are small (~1 mm long) buff-colored insects that crawl rapidly. They can usually be seen within newly opened flowers. Even strongly growing *Antirrhinum* are susceptible to infestation. This insect is both difficult to exclude from glasshouses and to treat with chemical insecticides, although biological control by the predatory mite *Neoseiulus* (formerly *Amblyseius*) *cucumeris* can keep infestation to a manageable level. The mite is available from biological control specialists.

Protocol 2

Propagating *Antirrhinum*

This protocol describes general strategies for propagating *Antirrhinum* species: self- and cross-pollination, cuttings, and grafting. *A. majus* cultivars and some wild species are self-fertile, but they require self-pollination for high seed yields. Although self-fertile, *A. majus* shows unilateral incompatibility and can only be crossed to other self-incompatible species as the female parent. All *Antirrhinum* species can be propagated clonally from cuttings. *Antirrhinum* also readily forms grafts within and between species.

MATERIALS

The recipe for the item marked with <R> is on page 117.

CAUTION: See the Cautions Appendix for appropriate handling of materials marked with <!>.

Reagents

Auxin-based rooting compound (for cuttings only; see Steps 7–10)

These compounds are often sold as “rooting hormone” and typically contain approximately 0.5% synthetic auxin. Gel-based solutions are easier and safer to handle than powders.

Compost <R> or washed sand (for cuttings only; see Steps 7–10)

Ethanol (70%) <!> (only if using forceps for pollination; see Steps 2 and 3.ii)

Plants, mature (see Protocol 1)

Equipment

Bags, mesh or glassine paper (for self- or cross-pollination only; see Steps 1–6)

Boxes, plastic, with silica desiccant (optional; see Step 6)

Covers, clear plastic, or mist propagation unit (for cuttings only; see Steps 7–10)

Forceps or toothpicks (for self- or cross-pollination only; see Steps 1–6)

Jewelry tags (for cross-pollination only; see Step 3.iii)

Lab film or transparent silicone rubber tubing (2–5-mm internal diameter) (for grafting only; see Steps 11–17)

Scalpel or razor blade (for grafting only; see Steps 11–17)

METHOD

Three separate procedures are presented here. For self- or cross-pollination, follow Steps 1–6; for cuttings, follow Steps 7–10; and for grafts, follow Steps 11–17.

Self- and Cross-pollination

1. Collect pollen from newly dehisced anthers by scraping the pollen from the anthers with forceps or a toothpick. For self-pollination, proceed to Step 2; for cross-pollination, proceed to Step 3.

The pollen should appear bright yellow and stick together in clumps. Older pollen that is lighter yellow and powdery is unlikely to be viable. To store pollen, freeze newly dehisced anthers at –80°C.

2. For self-pollination, use forceps or a toothpick to transfer pollen (see Step 1) to the stigma after the petals have opened. Between pollinations, remove pollen from the forceps with 70% ethanol. Discard used toothpicks or autoclave for reuse. Proceed to Step 4.

For rapid self-pollination, the lip of the lower petals can be used to smear pollen from anthers to stigma.

3. For cross-pollination, proceed as follows:
 - i. Emasculate a flower by removing the anthers from an unopened bud. Fold back the flower petals of the bud using forceps to expose the stamens. Remove the stamens by pulling the anther filaments away with forceps.

Most wild Antirrhinum species are self-incompatible and can only be pollinated by a plant carrying at least one different allele at the self-incompatibility locus. These species, therefore, do not require emasculation before cross-pollination.
 - ii. Using forceps or a toothpick, transfer pollen (Step 1) to the stigma of the emasculated flower (Step 3.i). Between pollinations, remove pollen from the forceps with 70% ethanol. Discard used toothpicks or autoclave for reuse.
 - iii. Label the crosses with jewelry tags looped around the peduncle.

4. Cover the inflorescences with mesh or glassine paper bags to prevent contamination with pollen from visiting bees.

Alternatively, exclude pollinating bees from the glasshouse or remove the lower three flower petals to prevent their use as a landing platform by bees.

5. Between 2 and 5 days after pollination, monitor the plants for abscission of the flower petals and stamens, which indicates that fertilization was successful. Remove any unfertilized flowers by cutting the peduncle close to the stem to prevent *Botrytis* infection of the inflorescence.
6. Once the pores in the fruit have dehisced (3–5 weeks after pollination), collect the seeds by shaking them out of the detached fruit.

The seeds will remain viable for several years under ambient conditions. For longer storage, seal the seeds in plastic boxes with silica desiccant at 4°C. Under these conditions (low temperature and low humidity), the seeds will remain viable for at least 10 years.

Cuttings

7. Cut a vegetative shoot 20–50 mm in length immediately below a leaf node. Cut off the leaves from the bottom two thirds of the shoot.
8. Dip the cut end in an auxin-based rooting compound and then insert it into compost or washed sand.
9. Grow the cutting at 17–20°C. Maintain high (~100%) humidity with a clear plastic cover or a mist propagation unit.
10. When new shoot growth is apparent (after 2–3 weeks), transplant to a larger pot of compost (see Protocol 1).

Grafts

Grafts of Young Plants

11. Graft young stems of similar diameter. Make slanting cuts in stock (recipient) and scion (donor) with a scalpel or razor blade. Cut off any large leaves from the scion to reduce water loss.

12. Hold the stock and scion together by binding them with lab film or by inserting them into opposite ends of a short length of transparent silicone rubber tubing.
13. Once the scion resumes growth, indicating a successful graft union, carefully cut away the lab film or tubing with a scalpel.

Grafts to Woody Stock

14. To graft to a large woody stock, make a T-shaped cut in the surface of the stem and ease back the soft green tissue with a scalpel blade.
15. Remove a vegetative axillary bud from the donor plant, together with a short length of leaf petiole, by cutting vertically through the soft stem tissue behind the bud.
16. Insert the donor bud (Step 15), in the correct orientation, into the T-shaped cut (in the stem from Step 14). Bind around the bud with lab film.
17. After 2–3 weeks, remove the film and cut the stock above the graft to promote bud growth.

Recipe

CAUTION: Horticultural chemicals and fertilizers may have potential health and environmental effects. Always follow manufacturer-recommended safety precautions and guidelines.

COMPOST

Ingredient	Quantity (per m ³)
Dolomitic limestone, powdered, to increase the pH to 5.5–6.5	4.0 kg
Fertilizer granules, slow-release, with an N:P:K ratio of ~20:10:20	1.0 kg
Fertilizer with micronutrients and an N:P:K ratio of ~15:10:20	0.75 kg
Horticultural wetting agent	according to supplier instructions
Peat or peat substitute	to 1 m ³
Perlite or vermiculite	150 liters

The fertilizer granules may be omitted, and instead, the established plants can be watered weekly with a soluble fertilizer. All *Antirrhinum* species are intolerant of waterlogged soil, particularly members of subsection *Kickxiella*, and an additional 20% (v/v) of washed sand or grit can be added to aid drainage. Avoid ammonium-based fertilizers, which encourage fungal wilting diseases.

REFERENCES

- Almeida, J. and Galego, L. 2005. Flower symmetry and shape in *Antirrhinum*. *Int. J. Dev. Biol.* **49**: 527–537.
- Bennett, M.D. and Leitch, I.J. 1995. Nuclear DNA amounts in angiosperms. *Ann. Bot.* **76**: 113–176.
- Carpenter, R. and Coen, E.S. 1995. Transposon induced chimeras show that *floricaula*, a meristem identity gene, acts non-autonomously between cell layers. *Development* **121**: 19–26.
- Cartolano, M., Castillo, R., Efremova, N., Kuckenberg, M., Zethof, J., Gerats, T., Schwarz-Sommer, Z., and Vandenbussche, M. 2007. A conserved microRNA module exerts homeotic control over *Petunia hybrida* and *Antirrhinum majus* floral organ identity. *Nat. Genet.* **39**: 901–905.
- Causier, B., Castillo, R., Zhou, J., Ingram, R., Xue, Y., Schwarz-Sommer, Z., and Davies, B. 2005. Evolution in action: Following function in duplicated floral homeotic genes. *Curr. Biol.* **15**: 1508–1512.
- Cubas, P. 2004. Floral zygomorphy, the recurring evolution of a successful trait. *Bioessays* **26**: 1175–1184.
- Cui, M.L., Handa, T., and Ezura, H. 2003. An improved protocol for *Agrobacterium*-mediated transformation of *Antirrhinum majus* L. *Mol. Genet. Genomics* **270**: 296–302.
- Darwin, C.R. 1868. *Variation of animals and plants under domestication*. John Murray, London.
- Davies, B., Cartolano, M., and Schwarz-Sommer, Z. 2006. Flower development: The *Antirrhinum* perspective. *Adv. Bot. Res.* **44**: 279–321.
- Egea-Cortines, M., Saedler, H., and Sommer, H. 1999. Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in *Antirrhinum majus*. *EMBO J.* **18**: 5370–5379.
- Feng, X., Zhao, Z., Tian, Z., Xu, S., Luo, Y., Cai, Z., Wang, Y., Yang, J., Wang, Z., Weng, L., et al. 2006. Control of petal shape and floral zygomorphy in *Lotus japonicus*. *Proc. Natl. Acad. Sci.* **103**: 4970–4975.
- Golz, J.F., Roccaro, M., Kuzoff, R., and Hudson, A. 2004. *GRAMINIFOLIA* promotes growth and polarity of *Antirrhinum* leaves. *Development* **131**: 3661–3670.
- Gübitz, T., Caldwell, A., and Hudson, A. 2003. Rapid molecular evolution of *CYCLOIDEA*-like genes in *Antirrhinum* and its relatives. *Mol. Biol. Evol.* **20**: 1537–1544.
- Hammer, K., Knüpffer, H., and Knüpffer, S. 1990. Das Gaterlebener Antirrhinum-sortiment. *Kulturpflanze* **38**: 91–117.
- Hileman, L.C., Kramer, E.M., and Baum, D.A. 2003. Differential regulation of symmetry genes and the evolution of floral morphologies. *Proc. Natl. Acad. Sci.* **100**: 12814–12819.
- Jiménez, J.F., Sánchez-Gómez, P., Güemes, J., Werner, O., and Rosselló, J.A. 2002. Genetic variability in a narrow endemic snapdragon (*Antirrhinum subbaeticum*, Scrophulariaceae) using RAPD markers. *Heredity* **89**: 387–393.
- Jiménez, J.F., Sánchez-Gómez, P., Güemes, J., and Rosselló, J.A. 2005. Phylogeny of snapdragon species (*Antirrhinum*; Scrophulariaceae) using non-coding cpDNA sequences. *Isr. J. Plant Sci.* **53**: 47–53.
- Keck, E., McSteen, P., Carpenter, R., and Coen, E. 2003. Separation of genetic functions controlling organ identity in flowers. *EMBO J.* **22**: 1058–1066.
- Lai, Z., Ma, W., Han, B., Liang, L., Zhang, Y., Hong, G., and Xue, Y. 2002. An F-box gene linked to the self-incompatibility (S) locus of *Antirrhinum* is expressed specifically in pollen and tapetum. *Plant Mol. Biol.* **50**: 29–42.
- Langlade, N.B., Feng, X., Dransfield, T., Copsey, L., Hanna, A.I.,

- Thebaud, C., Bangham, A., Hudson, A., and Coen, E. 2005. Evolution through genetically controlled allometry space. *Proc. Natl. Acad. Sci.* **102**: 10221–10226.
- Mateu-Andres, I. 2004. Low levels of allozyme variability in the threatened species *Antirrhinum subbaeticum* and *A. pertegasii* (Scrophulariaceae): Implications for conservation of the species. *Ann. Bot.* **94**: 797–804.
- Mateu-Andres, I. and de Paco, L. 2006. Genetic diversity and the reproductive system in related species of *Antirrhinum*. *Ann. Bot.* **98**: 1053–1060.
- McClure, B.A., Haring, V., Ebert, P.R., Anderson, M.A., Simpson, R.J., Sakijama, F., and Clarke, A.E. 1989. Style self-incompatibility gene products of *Nicotiana glauca* are ribonucleases. *Nature* **342**: 955–957.
- Nath, U., Crawford, B.C., Carpenter, R., and Coen, E. 2003. Genetic control of surface curvature. *Science* **299**: 1404–1407.
- Noda, K., Glover, B.J., Linstead, P., and Martin, C. 1994. Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor. *Nature* **369**: 661–664.
- Olmstead, R.G., dePamphilis, C.W., Wolfe, A.D., Young, N.D., Elisons, W.J., and Reeves, P.A. 2001. Disintegration of the Scrophulariaceae. *Am. J. Bot.* **88**: 348–361.
- Oyama, R.K. and Baum, D.A. 2004. Phylogenetic relationships of North American *Antirrhinum* (Veronicaceae). *Am. J. Bot.* **91**: 918–925.
- Rolland-Lagan, A.G., Bangham, J.A., and Coen, E. 2003. Growth dynamics underlying petal shape and asymmetry. *Nature* **422**: 161–163.
- Rothmaler, W. 1956. *Taxonomische monographie der gattung Antirrhinum*. Akademie Verlag, Berlin.
- Schwarz-Sommer, Z., de Andrade, S.E., Berndtgen, R., Lonnig, W.E., Muller, A., Nindl, I., Stuber, K., Wunder, J., Saedler, H., Gubit, T., et al. 2003. A linkage map of an F2 hybrid population of *Antirrhinum majus* and *A. molle*. *Genetics* **163**: 699–710.
- Schwinn, K., Venail, J., Shang, Y., Mackay, S., Alm, V., Butelli, E., Oyama, R., Bailey, P., Davies, K., and Martin, C. 2006. A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. *Plant Cell* **18**: 831–851.
- Serna, L. and Martin, C. 2006. Trichomes: Different regulatory networks lead to convergent structures. *Trends Plant Sci.* **11**: 274–280.
- Shang, Y., Schwinn, K.E., Bennett, M.J., Hunter, D.A., Waugh, T.L., Pathirana, N.N., Brummell, D.A., Jameson, P.E., and Davies, K.M. 2007. Methods for transient assay of gene function in floral tissues. *Plant Methods* **3**: 1.
- Stubbe, H. 1966. *Genetik und zytologie von Antirrhinum L. sect. Antirrhinum*. Gustav Fischer, Jena.
- Webb, D.A. 1972. *Antirrhinum L.* In *Flora europaea* (ed. T.G. Tutin, et al.), pp. 221–224. Cambridge University Press, New York.
- Whibley, A.C., Langlade, N.B., Andalo, C., Hanna, A.I., Bangham, A., Thebaud, C., and Coen, E. 2006. Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science* **313**: 963–966.
- Wright, G.A., Lutmerding, A., Dudareva, N., and Smith, B.H. 2005. Intensity and the ratios of compounds in the scent of snapdragon flowers affect scent discrimination by honeybees (*Apis mellifera*). *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **191**: 105–114.
- Yang, Q., Zhang, D., Li, Q., Cheng, Z., and Xue, Y. 2007. Heterochromatic and genetic features are consistent with recombination suppression of the self-incompatibility locus in *Antirrhinum*. *Plant J.* **51**: 140–151.
- Zhang, D., Yang, Q., Bao, W., Zhang, Y., Han, B., Xue, Y., and Cheng, Z. 2005. Molecular cytogenetic characterization of the *Antirrhinum majus* genome. *Genetics* **169**: 325–335.

FURTHER READING

- Schwarz-Sommer, Z., Davies, B., and Hudson, A. 2003. An everlasting pioneer: The story of *Antirrhinum* research. *Nat. Rev. Genet.* **4**: 657–666.
Reviews the history of *Antirrhinum* genetics.
- Davies, B., Cartolano, M., and Schwarz-Sommer, Z. 2006. Flower development: The *Antirrhinum* perspective. *Adv. Bot. Res.* **44**: 279–321.
Provides a detailed review of the control of flower development in *Antirrhinum*, in comparison to that of other species.

WWW RESOURCES

- <http://www.antirrhinum.net> DragonDB homepage. The *Antirrhinum majus* (snapdragon) genetic and genomic database. The contents of this searchable database in AceDB schema include *Antirrhinum* sequences, mutants, maps, and publications.
- <http://www.jic.ac.uk/STAFF/enrico-coen/Rosemary/start.htm> *Antirrhinum* stock collection, The John Innes Centre.

16

The Sea Lamprey *Petromyzon marinus* A Model for Evolutionary and Developmental Biology

Natalya Nikitina, Marianne Bronner-Fraser, and Tatjana Sauka-Spengler
Division of Biology, California Institute of Technology, Pasadena, California 91125

ABSTRACT

Sea lampreys (*Petromyzon marinus*) are cyclostomes, the most basal extant group of vertebrates, and are thought to have existed largely unchanged for more than 500 million years. They are aquatic, eel-shaped animals that spend a major part of their life as filter-feeding larvae called ammocoetes, inhabiting many freshwater bodies in the northern hemisphere. After metamorphosis, sea lampreys migrate to the ocean (or to the Great Lakes), where they feed on the blood and bodily fluids of salmonid fish and ultimately return to freshwater streams and rivers to spawn and die. The unique evolutionary position of lampreys and the relative ease of obtaining mature adults and embryos make this animal an ideal model for investigations into early vertebrate evolution. Studies of features shared between lampreys and jawed vertebrates, but distinct from those in nonvertebrate chordates, have informed on the origin and evolution of hallmark vertebrate characteristics such as the neural crest, ectodermal placodes, and jaw. In addition, studies of features that are unique to lampreys (e.g., the variable lymphocyte receptor [VLR]-mediated immune system) provide insights into mechanisms of parallel evolution (e.g., the adaptive immune system). With the establishment of techniques for the extended maintenance and spawning of lampreys in the laboratory, the sequencing of the lamprey genome, and the adaptation and optimization of many established molecular biology and histochemistry techniques for use in this species, *P. marinus* is poised to become an evolutionary developmental model of choice.

PROTOCOLS

- 1 Culturing Lamprey Embryos, 410
- 2 Microinjection of RNA and Morpholino Oligos into Lamprey Embryos, 413
- 3 Dil Cell Labeling in Lamprey Embryos, 415
- 4 Whole-mount In Situ Hybridization on Lamprey Embryos, 417
- 5 Immunostaining of Whole-mount and Sectioned Lamprey Embryos, 421

BACKGROUND INFORMATION

Petromyzon marinus (commonly known as sea lamprey but sometimes called lake lamprey or lamprey eel) is an aquatic animal with an elongated snake-like body reaching, on average, 45–60 cm in length and weighing up to 900 g. Adults are mottled brown in color with a lighter ventral side that becomes yellow during the breeding season.

Lamprey eggs are laid in freshwater streams during the summer, and after spawning, the adults usually die. The embryos hatch after about 2 weeks, and the larvae (called ammocoetes) spend the first 7–9 years of their life burrowed in mud in freshwater streams, filter-feeding on small mud-inhabiting organisms. After this period of time, ammocoetes undergo an extensive metamorphosis, during which they develop suckorial discs, eyes, and dorsal fins, and they migrate into the ocean, where they spend the next 18–24 months feeding on fish blood. When lampreys reach their full adult size, they become sexually mature, stop feeding, and, like other anadromous fish, migrate back to the streams and rivers where they hatched to repeat the cycle of reproduction and death (Hardisty and Potter 1971b; Hardisty 1979).

Sea lampreys are generally marine, inhabiting the Atlantic Ocean and migrating into freshwater rivers and streams of Europe and the United States Atlantic coast to reproduce. The U.S. also has two landlocked populations of sea lampreys, one in the Great Lakes and the other in Lake Champlain. These populations originated in 1835 when lampreys were inadvertently introduced into Lake Ontario. It is quite possible that they migrated by attaching themselves to the hulls of ships going through the canal systems of Lake Erie or the St. Lawrence River. By 1946, sea lampreys had spread throughout the Great Lakes and the freshwater streams of the Great Lakes region. Because these landlocked lampreys have not been able to enter the ocean to feed on marine fish, they have become parasitic, preying on freshwater fish and causing considerable damage to the ecosystem, which has necessitated government-controlled measures. Freshwater lampreys inhabiting the Great Lakes are distinct from Atlantic lampreys in size and color, with the former being smaller and more brown and yellow in color and the latter much larger and silvery in color.

SOURCES AND HUSBANDRY

Each year, from late April through mid-July, adult sea lampreys are collected from tributaries of Lake Huron and Lake Michigan by the U.S. Fish and Wildlife Service Marquette Biological Station (Marquette, Michigan). The maturing adults, migrating upstream, are trapped, and spawning adults are removed directly from their nests. After collection, the animals are transported to laboratories at the U.S. Geological Survey Hammond Bay Biological Station (Millersburg, Michigan). Adult prespermiating males and preovulating females are then shipped to our laboratory where they are kept in a custom-made lamprey aquatic housing system (Aquaneering).

This housing system features four durable, insulated, polypropylene tanks, each with an individual tank valve for precisely metering the water flow rates. The system also features a mechanism for aeration and uses a four-stage filtration system that includes a fluidized bed biological filter and single-canister charcoal filter. Wastewater first flows through a Dacron pad mechanical filter, trapping particles greater than 10 μm , and then through the fluidized bed biological filter. The fluidized bed biological filters are composed of a fine medium that provides a large surface area on which hundreds of different types of beneficial bacteria can grow. This ensures a biologically stable ecology in the tank system that has undetectable levels of ammonia and nitrites. Each tank has an individual temperature- and light-control system that can be set to manually or automatically manipulate the temperature and the light/dark cycle in the lamprey rack. Each also has an automatic water-exchange system that can be programmed to perform the daily change-out of water conditioned to the proper pH and salinity levels. In our laboratory, sea lampreys are held under conditions that mimic their natural cycle until they reach the spermiated/ovulating phase, when they are used for artificial fertilization.

RELATED SPECIES

Thirty-eight lamprey species have been described; four species inhabit the southern hemisphere and have been placed into the families Geotriidae and Mordaciidae, whereas the 34 remaining

species are all from the northern hemisphere and have been assigned to six genera that are grouped together into one family, Petromyzontidae (Hubbs and Potter 1971). The phylogenetic relationships among the various lamprey genera have not yet been adequately resolved, but molecular phylogenetic analyses indicate that lampreys are paraphyletic in origin.

Three lamprey species, *P. marinus*, *Lampetra (Lethenteron) japonica*, and *L. fluviatilis* (European river lamprey), have historically been the “stars” of lamprey research; they remain, to this day, the preferred lamprey research models. These lampreys are relatively large, produce huge quantities of eggs, are widely distributed, and are easy to capture when they migrate into freshwater streams at the start of the breeding season. *L. japonica* is used by Japanese researchers; *L. fluviatilis* is studied in Europe; and *P. marinus*, the focus of this chapter, is a preferred model in European and North American labs. All of the techniques for laboratory maintenance, embryo culture, and embryo manipulation described here are applicable to all three species, although a different embryo culture medium (10% Steinberg’s solution) has been used successfully with *L. japonica* embryos (Horigome et al. 1999).

USES OF THE *P. MARINUS* MODEL SYSTEM

The sea lamprey has been studied since the mid-19th century. At that time, lampreys were thought to be either primitive, degenerate vertebrates or direct ancestors of modern vertebrates. As a consequence, many of the first studies focused on comparing the morphology of the adult lampreys to that of higher vertebrates in an attempt to clarify the taxonomic position of this animal group (Huxley 1876). Adult lampreys used in these studies were wild-caught; the first attempt to maintain lamprey larvae in captivity was reported in 1900 (Reese 1900). Techniques for spawning, in vitro fertilization, and short-term maintenance of lamprey were first established in related species from the genus *Lampetra*, which allowed for a detailed analysis of early embryonic development to be performed in this species (Damas 1944). The embryonic development of *P. marinus* was first described by George Piavis (Piavis 1961, 1971); his developmental staging system is still widely used. More recently, Tahara (1988) described developmental stages for *Lampetra reissneri* and correlated them with Piavis stages, creating the developmental table that is probably the most commonly used today.

With the advent of modern molecular techniques, lamprey became one of the most important research models for understanding vertebrate origins (for review, see Kuratani et al. 2002; Osorio and Retaux 2008). Lampreys, together with hagfish, are the most basal animals in which many of the true vertebrate characteristics (e.g., neural crest, placodes, segmented brain, skull, paired sensory organs, pharyngeal skeleton) are present. By studying the molecular and developmental mechanisms responsible for the formation of these structures in lamprey and higher vertebrates, we can gain insight into how these vertebrate characteristics evolved.

Here, we concentrate on the aspects of lamprey biology that are most relevant to developmental and evolutionary biology. Readers interested in a more comprehensive description of lamprey physiology, morphology, behavior, and ecology may refer to *The Biology of Lampreys* (Hardisty and Potter 1971a,b; 1971–1982).

Embryonic Development

An excellent description of sea lamprey development was provided by Piavis (1971), who divided the embryonic developmental pattern into 18 stages—from fertilized egg to ammocoete—based on easily discernable external characteristics. Cleavage is holoblastic; animal blastomeres are visibly smaller than vegetal blastomeres. Gastrulation (Piavis stage 9 or 64–100 hours postfertilization [hpf]) occurs by a mechanism that is very similar to that of *Xenopus* gastrulation, and the first signs of neural plate formation are seen at 4.5 days of development, with the thickened layer of the neural ectoderm on the dorsal aspect of the embryo and the flattened neural plate border clearly

distinguishable from the surrounding ectoderm. The raised edges of the neural plate fuse to form a solid neural rod that later cavitates to form a hollow neural tube. The embryos begin to move inside their chorions by days 9–10 (Piavis stage 13). On day 11 (Piavis stages 13–14), they hatch, their hearts start to beat, and gill clefts form and start to function by day 17 (Piavis stage 15). Eye development in the lamprey proceeds in a unique fashion: The formation of the eye primordium, with an undifferentiated retina and lens, is accomplished during embryonic life (days 17–20; Piavis stages 15–16), but the growth of the eye and the maturation of the retinal receptors arrest during the long larval life and resume only after metamorphosis (Rubinson 1990).

Lamprey is one of the slowest-developing vertebrates, and its embryos develop abnormally and often arrest in development at temperatures even slightly below the optimal temperature of 18°C. Thus, the pace of their development cannot be adjusted by modulating the rearing temperature.

Immunology

It has been known since the mid-20th century that lampreys possess an efficient adaptive immune system (for review, see Cooper and Alder 2006); they demonstrate accelerated rejection of secondary skin allografts and increased agglutination after repeated immunization with particulate antigens (e.g., anthrax bacteria, sheep blood cells). Interestingly, lampreys do not appear to respond to soluble antigens. Despite the presence of lymphocytes and the obvious ability to mount an immune response, lampreys do not possess either immunoglobulin-type or T-cell receptors, which are the hallmark of the adaptive immune systems of higher vertebrates. Instead, lampreys rely on a completely different type of variable lymphocyte receptor (VLR) that is composed of a highly variable leucine-rich repeat (LRR) and a stalk segment of conserved sequence that connects the VLR to the surface of the lymphocyte (Pancer et al. 2004). Like the vertebrate immunoglobulin-type receptors, diverse VLRs are generated in lymphocytes by genome recombination. A combination of variable gene segments, including many LRRs, is assembled into a VLR, and the intervening genomic sequence is permanently excised from the genome of that lymphocyte lineage (Nagawa et al. 2007). Vast numbers of lymphocyte receptors, rivaling the diversity of the mammalian immune repertoire, are produced in this way (Alder et al. 2005).

Lampreys do not possess an anatomical equivalent of the mammalian thymus. In ammocoetes, blood cell formation and lymphocyte maturation occur in the typhlosole (an invagination of the intestinal epithelium) and the nephric fold (Amemiya et al. 2007). In adult lampreys, lymphocyte formation occurs in the gills and the ventral kidney.

Phylogeny

Phylogenetic relationships among the lampreys, hagfishes, and gnathostomes have been the subject of many molecular analyses, and the current consensus, based on molecular analyses of ribosomal RNA and nuclear genes, is that the lampreys and hagfishes form a monophyletic sister group to the craniates (Stock and Whitt 1992; Takezaki et al. 2003). The 38 currently recognized species of extant lampreys are considered to be paraphyletic in origin and are grouped into three families on the basis of morphological characteristics, including dentition and the shape of the suctorial disc (Gill et al. 2003). From an ecological perspective, lampreys can be divided into three groups on the basis of their feeding strategies as adults: anadromous lampreys, freshwater parasitic lampreys, and brook lampreys. Anadromous lampreys, which includes the two species most commonly used for research (*P. marinus* and *L. japonica*), spend their adult lives in saltwater habitats, feeding on the blood of marine fish. Parasitic freshwater species spend their entire lives in the streams and rivers in which they hatch and, due to the more limited food supply, they tend to be smaller than their anadromous relatives and have a shorter adult stage. Twenty of the extant lamprey species are dwarf, nonparasitic brook lampreys, which never feed after metamorphosis. The brook lampreys most likely evolved from parasitic freshwater ancestors by delaying metamorphosis and shortening the adult stage (Hardisty and Potter 1971a).

GENETICS, GENOMICS, AND ASSOCIATED RESOURCES

The sequencing of the 2.1–2.4-Gb genome of *Petromyzon marinus*, financed by the National Human Genome Research Institute (NHGRI), is well under way, and the project aims to produce a high-quality assembly. So far, the genome has been sequenced to 5.9x whole-genome coverage, and the current assembly primarily consists of whole-genome, shotgun, plasmid-end sequences, with a small sampling (0.005x) of fosmid-end sequences. This assembly can be viewed and downloaded from the website maintained by Washington University in the St. Louis Genome Sequencing Center (<http://genome.wustl.edu/genome.cgi?GENOME=Petromyzon%20marinus&SECTION=assemblies>), and raw sequence data in NCBI's Trace Archive can be searched using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PAGE=Nucleotides&PROGRAM=ST_SPEC=TraceArchive&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch).

A bacterial artificial chromosome (BAC) library using the genomic material of the specimen being sequenced was constructed, arrayed, and spotted onto nylon macroarray filters, which are available from BACPAC Resources, Children's Hospital Oakland Research Institute (Oakland, CA; <http://bacpac.chori.org/library.php?id=199>). Because initial studies indicated that the genome is A+T-rich and repetitive and that the heterozygosity rate for a single animal is very high, additional sequencing of BAC and fosmid ends is planned. Conventional expressed sequence tag (EST) sequencing of cDNA libraries is also under way; this should help with gene annotation and will provide clones of interest for the community. Because the sequencing project is ongoing, the quality of the assembly should improve and the number of partially or fully sequenced cDNA clones from different stages and tissue-specific libraries will increase.

TECHNICAL APPROACHES

The following protocols describe some basic techniques for culturing and studying lamprey embryos in the laboratory.

Protocol 1

Culturing Lamprey Embryos

This protocol describes how to produce lamprey embryos by collecting sperm and eggs from mature lamprey, performing fertilization, and culturing the embryos through to the desired developmental stage. The embryos produced in this protocol can be used for all of the investigative procedures described in Protocols 2–5.

MATERIALS

The recipe for the item marked with <R> is on page 427.

Reagents

H₂O, distilled and spring (Sparkletts) (both equilibrated to 18°C)

Lampreys (mature spermiating males and ovulating females)

Obtain lampreys and maintain actively spermiating males (Fig. 1A) and ovulating females (Fig. 1B) in an aquarium as described in the “Sources and Husbandry” section. Because lampreys tend to die after spawning, we usually maintain the actively spermiating males and ovulating females at 12°C in order to extend their lives and slow down their natural tendency to die.

0.1x MMR, equilibrated to 18°C <R>

Equipment

Crystallization dishes

Incubator, preset to 18°C



FIGURE 1. (A) Mature spermiating males are recognizable by their prominent dorsal ridge. (B) Ovulating females lack the dorsal ridge but have enlarged, soft abdomens. (C) In vitro fertilization is performed in spring water at 18°C. Eggs are obtained by gently squeezing the abdomen of a mature female and are then deposited into a crystallization dish. Subsequently, sperm from mature males are expressed in a similar manner and are added to the dish. The dish of eggs and sperm is gently swirled several times and then allowed to rest for about 15 minutes.

METHOD

1. Obtain eggs by gently squeezing the abdomens of gravid females (Fig. 1C). Deposit the eggs into a crystallization dish containing approximately 250–350 ml of spring H₂O. Obtain sperm from spermiating males using the same technique.

Mature females (Fig. 1B) can be distinguished from immature specimens by their distended soft abdomen and the absence of a dorsal ridge, whereas mature males (Fig. 1A) have a very thick, prominent, raised dorsal ridge (Vladykov 1949). See Troubleshooting.

2. To fertilize the eggs, add the sperm to the crystallization dish, swirl the solution of sperm and eggs several times, and allow the dish to stand for at least 15 minutes.

To allow cortical rotation to occur normally, it is important to avoid moving the dish with fertilized eggs too much immediately after adding sperm. The success of fertilization can be monitored by observing the fraction of eggs whose fertilization envelopes lift.

3. Wash out the excess sperm with four changes of distilled H₂O. Do so gently to avoid introducing air bubbles.

Air bubbles adhere to the membranes of the eggs, causing the eggs to float and the embryos to be lost during the washes.

4. Replace the distilled H₂O with fresh spring H₂O and incubate the embryos at 18°C until the first cell division is under way, which normally takes about 6–6.5 hours.

See Troubleshooting.

5. Before cleavage (within 10 hours of fertilization), replace the spring water with sterile 0.1× MMR.

MMR contains CaCl₂. Cleaving embryos require Ca²⁺ in order to form intercellular junctions and will die if left in spring H₂O for more than 10 hours.

6. Incubate the embryos in the incubator until the desired developmental stage is reached. On a daily basis, inspect the culture dishes, remove any dead or arrested embryos, and replace the 0.1× MMR. To avoid fungal infections and massive embryonic death, carefully maintain the embryos in sterile, nonconfluent conditions. After the third day of incubation, ensure that the embryos are spread over the dish such that every embryo is at least 1 cm away from its neighbors. Leave the embryos undisturbed until day 4.5.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 1): Females do not release eggs easily or blood is present during the extraction procedure.

Solution: The females have not yet matured. Allow them to develop for another 2–4 days at 18–19°C before attempting to extract eggs again.

Problem (Step 4 or 6): Abnormal cleavage or arrested embryonic development.

Solution: Consider the following:

- Abnormal cleavage and arrested development are evidence of polyspermy, so avoid using too much sperm, especially in June or early July. The quality of sperm progressively decreases throughout the mating season. In August, use sperm from several males in order to ensure complete fertilization.

- Ensure the highest egg quality by obtaining immature female lampreys and allowing them to develop for 2–3 weeks before extracting their eggs. Do not use older females that are already ovulating.
- Wash the eggs extensively after fertilization (Step 3).
- Ensure that the incubator temperature does not fluctuate from 18°C.

Problem (Step 6): Embryos do not survive past days 4–5 of development.

Solution: Disturbing or moving embryos between days 3–5 of incubation can result in massive embryonic death. On day 3, be sure that each embryo is at least 1 cm away from its neighbors, and leave it undisturbed until gastrulation is completed (day 4.5). During this vulnerable stage, embryos can be raised individually in 48-well plates.

Problem (Step 6): Embryos cleave and gastrulate normally, but show slowed development at later stages (i.e., the head does not begin to form by day 5).

Solution: Ensure that spermiating males and ovulating females are not exposed to temperatures below 10°C. Lower temperatures seem to affect the viability of the eggs.

Protocol 2

Microinjection of RNA and Morpholino Oligos into Lamprey Embryos

Lamprey embryos are particularly amenable to injection techniques. They have the same advantages as both zebrafish and *Xenopus* embryos in that, due to their double chorion, they are not prone to surface-tension-induced explosion when removed from liquid and can therefore be injected in a dry dish. This eliminates the need to support the embryo while performing injections, making the procedure very rapid. Also, a single ovulating female may contain up to 100,000 eggs, so the numbers of injectable embryos per fertilization is not a limiting factor, which is a great advantage over *Xenopus* and zebrafish. Finally, the second division lasts for several hours, providing a very large injection window. This protocol describes how to microinject RNA and morpholinos into lamprey embryos using previously described techniques (McCauley and Bronner-Fraser 2006; Sauka-Spengler et al. 2007).

MATERIALS

The recipe for the item marked with <R> is on page 427.

CAUTION: See the Cautions Appendix for appropriate handling of materials marked with <!>.

Reagents

Lamprey embryos (at the two-cell stage; see Protocol 1)

0.1x MMR, equilibrated to 18°C <R>

Morpholino oligos (FITC <!> labeled; e.g., Gene Tools)

Sense RNA (100 µg/µl)

The full-length sense RNA for the gene of interest can be prepared by in vitro transcription (e.g., using one of Ambion's mMessage mMachines kits). Before injection, a small amount of RNase-free vegetable dye should be added to the RNA solution to assist with visualizing the injection (the vegetable dye is not required for the FITC-labeled morpholinos because their slightly yellow color allows direct visualization).

Equipment

Glass needles, fine-pulled

Incubator, set at 18°C

Injection dish

Prepare an injection dish by gluing a fine nylon mesh (Sefar Filtration Inc.) to the bottom of a standard Petri dish. Use a few drops of acetone <!> to bind the mesh to the dish.

Micrometer

Microscope (dissecting)

Microscope (fluorescent)

Picospritzer

Transfer pipettes (wide bore, plastic)

METHOD

1. Use a wide-bore, plastic, transfer pipette to transfer 100–200 two-cell-stage embryos (Fig. 2A) into an injection dish. Choose viable embryos that are cleaving normally; avoid those that are not dividing or are dividing irregularly.
2. Remove the liquid from the dish and allow the embryos to settle into the holes in the mesh.
3. Back-fill the glass needle with 6–10 μ l of morpholino (or sense RNA) solution. For each two-cell-stage embryo, inject about 5 nl into one blastomere. Use the dissecting microscope to aid in visualization.

The exact volume required should be determined by a standard calibration method or by measuring the droplet diameter with the help of a micrometer. As a rough guide, the diameter of the drop that is injected should not exceed about one-fourth the diameter of a single blastomere. For most embryos, this volume corresponds to approximately 5 nl of aqueous injection solution. If required, adjust the picospritzer until the desired drop size is achieved. See Troubleshooting.

4. Incubate the injected embryos at 18°C in 0.1X MMR until the desired developmental stage is reached.

See Protocol 1 for general information on culturing lamprey embryos.

5. When the embryos reach the desired stage of development, use the fluorescent microscope to select those that integrated the morpholino on one side only (Fig. 2B).

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 3): The injection needle is blocked.

Solution: Clean or replace the needle. The morpholino solution and egg cytoplasm have a tendency to block the needle, so it should be checked regularly and cleaned or replaced as necessary.

Problem (Step 5): The injected agent leaks into the noninjected side of the embryo.

Solution: Leakage occurs when two-cell-stage embryos are injected because, in lamprey, the first cell division is not completed until the middle of the second cell division. To avoid leakage, either (1) inject two blastomeres at the four-cell stage or (2) inject during the middle of the second cell division. If you choose the latter, perform the injection at the end of the dividing blastomere, opposite the furrowing site, to ensure an even distribution of the injected agent throughout the dividing cell. This approach will necessarily narrow the injection time window.

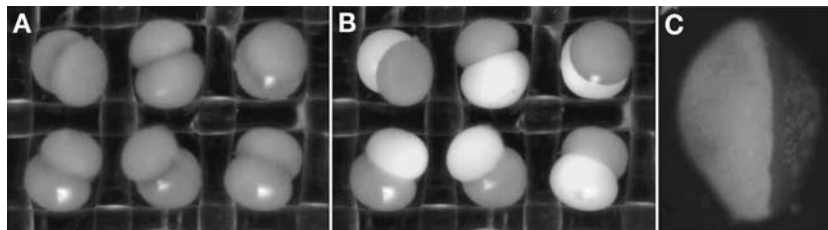


FIGURE 2. (A) Lamprey embryos at the two-cell stage. (B) Embryos injected with an FITC-labeled morpholino antisense oligo. Each injection was into a single blastomere at the two-cell stage. (C) Dorsal view of a 5-day-old neurula, which had been injected at the two-cell stage, showing the unilateral integration of the fluorescent morpholino.

Protocol 3

DiI Cell Labeling in Lamprey Embryos

This protocol describes how to label lamprey embryo cells by microinjecting the fluorescent dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) to study cell fate during development. It was adapted from McCauley and Bonner-Fraser (2003).

MATERIALS

The recipes for the items marked with <R> are on page 427.

CAUTION: See the Cautions Appendix for appropriate handling of materials marked with <!>.

Reagents

DiI (0.5 µg/µl), prepared in 0.3 M sucrose
Lamprey embryos (5–6 days old; see Protocol 1)
1× MMR <R>
Paraformaldehyde (4%) <!>
10× Phosphate-buffered saline (PBS) <R>, diluted to 1× before use

Equipment

Dissecting microscope
Fluorescence microscope
Forceps (sharp; two pairs)
Glass electrodes (pulled)
Incubator, preset to 18°C
Petri dishes
Coat some dishes with 1% agarose and create 1-mm depressions that will hold the embryos in place (see Step 2).
Picospritzer
Vibratome (optional; see Step 8)

METHOD

1. Submerge the 5–6-day-old embryos in 1× MMR. Remove the chorions from the embryos using sharp forceps. Use a dissecting microscope to aid in visualization.
The 1× MMR solution is used during chorion removal and for subsequent culturing to prevent the embryos from exploding.
2. Place the dechorionated embryos into 1-mm depressions in the agarose-coated Petri dishes.
3. Back-fill the glass electrodes with the DiI solution. Using the picospritzer, inject a single bolus under the ectoderm to label a discrete region of the embryo (e.g., the dorsal neural tube).

4. View the injected embryos under a fluorescence microscope to confirm that the labeling is confined to a single discrete location.
5. Transfer the embryos to Petri dishes containing 1X MMR. Incubate the dishes at 18°C until they reach the desired stage of development.

See Protocol 1 for general information on culturing lamprey embryos.

6. Once the embryos have reached the desired age, replace the 1X MMR with 4% paraformaldehyde and incubate for 1–2 hours at room temperature to fix the tissue.
7. Rinse the embryos thoroughly with room-temperature 1X PBS to remove the fixative.
8. Examine the embryos under the fluorescence microscope to observe the final destinations of the labeled cells (e.g., the neural crest cells).

To determine the location of the labeled cells with greater accuracy, the embryos can be embedded in 5% agarose and sectioned with a vibratome.

Protocol 4

Whole-mount In Situ Hybridization on Lamprey Embryos

This protocol describes an optimized procedure for RNA in situ hybridization in lamprey embryos. It is based on the protocol previously described by Sauka-Spengler et al. (2007).

MATERIALS

The recipes for the items marked with <R> begin on page 425.

CAUTION: See the Cautions Appendix for appropriate handling of materials marked with <!>.

Reagents

Anti-DIG FAB fragments (alkaline-phosphatase conjugated [1:2000 dilution], prepared in blocking solution)

If using FITC <!>-labeled probes, use anti-FITC FAB fragments instead of anti-DIG FAB fragments, conjugated to alkaline phosphatase.

Bleaching solution (freshly prepared) <R>

Blocking solution <R>

Color development solution <R>

Glycerol (75%, prepared in PBST)

To prevent mold growth, include 0.2% sodium azide in the 75% glycerol solution.

Glycine (2 mg/ml, freshly prepared in PBST-DEPC)

Hybridization mix <R>

Hybridization mix:MABT (1:1)

Lamprey embryos at desired developmental stage (see Protocol 1)

1× MABT <R>

MEMFA <R>

Methanol <!>

NTMT <R>

Paraformaldehyde (4%) <!>

Proteinase K (14–22 mg/ml) <!>, prepared in PBST-DEPC

PBST <R>

In addition, prepare three separate solutions of PBST containing 25, 50, and 75% methanol.

PBST-DEPC <R>

In addition, prepare three separate solutions of PBST-DEPC containing 25, 50, and 75% methanol.

Postfix solution <R>

RNA probe (DIG labeled; final concentration 2–4 µg/ml)

Both antisense and sense (control) probes should be synthesized for the genes of interest using a standard in vitro transcription kit containing DIG-labeled nucleotides. FITC-labeled probes can also be used.

Equipment

Aluminum foil

Forceps (sharp, two pairs)

Hybridization oven set at 70°C

Light box
 Microcentrifuge tubes (2 ml, transparent)
 Microscope (fluorescence)
 Nutator

METHOD

All steps should be performed at room temperature unless otherwise noted. Throughout the protocol, use approximately 2 ml of each solution for each group of 20–30 embryos. All steps should be performed using a nutator, except where specifically mentioned otherwise.

Fixation and Dehydration

1. Place embryos in a 2-ml microcentrifuge tube. Fix the embryos in MEMFA for 1 hour with shaking on the nutator.
2. Wash the embryos in PBST-DEPC for 15 minutes with shaking on the nutator. Discard the solution. Repeat this step three more times.
3. With shaking on the nutator, wash the embryos for 15 minutes in each of the following solutions:
 - PBST-DEPC containing 25% methanol
 - PBST-DEPC containing 50% methanol
 - PBST-DEPC containing 75% methanol
 - 100% methanol

This slow, gradual dehydration of embryos is important to prevent the chorions from sticking to the surface of the embryo.

4. Rinse the embryos several times in methanol.
 - At this point, embryos can be stored for up to several years at –20°C.*
5. With shaking on the nutator, wash the embryos for 30 minutes in each of the following solutions:
 - Once with PBST-DEPC containing 75% methanol
 - Once with PBST-DEPC containing 50% methanol
 - Once with PBST-DEPC containing 25% methanol
 - Three times with PBST-DEPC

This slow rehydration procedure ensures that the chorions separate from the surface ectoderm to facilitate chorion removal postfixation. Although lamprey chorions are softer and easier to remove manually before the embryos are fixed, it is not practical to remove the chorions from the embryos at early stages of development (before days 5.5–6) because the embryos are very fragile. (Also, we have found that enzymatic dechoriation methods do not work in lamprey.)

Dechoriation and Pretreatment

6. Remove the chorions from the embryos using forceps.
 - After chorion removal, embryos can be dehydrated and stored at –20°C, once again, as described in Steps 3 and 4. When being brought out of storage, wash the embryos three times for 5–10 minutes each in PBST-DEPC before proceeding with Step 7.*
7. Replace the PBST-DEPC with freshly made bleaching solution. Place the tubes containing the embryos on the light box for 7–10 minutes.

This bleaching step increases the signal in the detection step. If a stronger signal is desired, the incubation time can be increased to 15 minutes, but overbleaching will damage the ectoderm in younger embryos. Four-day embryos should not be subjected to bleaching for more than 7 minutes. Note that the embryos must be in transparent tubes to allow the light to penetrate.

8. Rinse the embryos three times for 5 minutes each with PBST-DEPC.
9. Treat the embryos with 14–22 $\mu\text{g/ml}$ proteinase K in PBST-DEPC at room temperature. Incubation time depends on the age of the embryo:
 - For 4.5-day embryos and younger, incubate for 5 minutes.
 - For 4.5–6-day embryos, incubate for 7–8 minutes.
 - For 6-day and older embryos, incubate for 9–10 minutes.

The proteinase K treatment makes the embryos very fragile, so they should not be agitated until they are refixed. Therefore, do not use the nutator from now until Step 11.
10. Replace the proteinase K solution with freshly prepared 2 mg/ml glycine. Incubate the embryos for 10 minutes.
11. Rinse the embryos twice for 5 minutes each with PBST-DEPC.
12. Incubate the embryos in postfix solution for 20 minutes with mixing on the nutator.
13. Rinse the embryos four times for 5 minutes each with PBST-DEPC.
14. Wash the embryos two or three times for 10 minutes each with hybridization mix.

At this point, the embryos can be stored indefinitely at -20°C in hybridization mix.

Prehybridization and Hybridization

15. Add fresh hybridization mix to the embryos and prehybridize by incubating for at least 3 hours at 70°C in the hybridization oven.
16. Add fresh, prewarmed (70°C) hybridization mix containing 1–10 $\mu\text{l/ml}$ labeled RNA probe. To hybridize the probe, incubate the embryos overnight (or for at least 16 hours) at 70°C in the hybridization oven with agitation on the nutator.

Posthybridization Washes

17. Remove the hybridization mix containing the probe and store it at -20°C for reuse.

Probes can be reused five to eight times.
18. Using prewarmed (70°C) hybridization mix, wash the embryos in the 70°C hybridization oven as follows:
 - Twice for 15 minutes each (change the hybridization mix after each wash)
 - Four times for 30–45 minutes each (change the hybridization mix after each wash)
19. Wash the embryos with hybridization mix:MABT (1:1) for 30 minutes in the 70°C hybridization oven.
20. Wash the embryos four times with MABT for 30 minutes each at room temperature.

Blocking and Color Development

21. Block the embryos in blocking solution for 1 hour at room temperature.
22. Add the diluted anti-DIG-alkaline phosphatase antibody solution to the embryos and shake on the nutator overnight at 4°C .
23. Wash the embryos with MABT as follows:
 - Twice for 5 minutes each at room temperature
 - Eight times for 30–60 minutes each at room temperature
 - Overnight at 4°C
24. Wash the embryos four times with NTMT for 15 minutes each at room temperature.

25. Incubate the embryos in color development solution in the dark (e.g., by covering in aluminum foil) for 1–72 hours.

The incubation should continue until the color has developed, which will depend on the abundance of the target RNA in the tissue. Slow-developing embryos (those being stained for RNA that is expressed at very low levels) can be incubated overnight.

Postcolor Washes and Postfix

26. Wash the embryos three times with PBST for 5 minutes each in the dark.
27. Incubate the embryos in 4% paraformaldehyde for 2 hours at room temperature.
Alternatively, incubate the embryos in 4% paraformaldehyde overnight at 4°C.
28. Wash the embryos three times with PBST for 10 minutes each.
29. With shaking on the nutator, wash the embryos as follows:
 - With PBST containing 25% methanol for 15 minutes
 - With PBST containing 50% methanol for 15 minutes
 - With PBST containing 75% methanol for 15 minutes
 - With 100% methanol for 2–3 hours

This step removes background staining. At this stage, embryos can be stored at –20°C.
30. With shaking on the nutator, wash the embryos for 10 minutes in each of the following solutions:
 - Once with PBST containing 75% methanol
 - Once with PBST containing 50% methanol
 - Once with PBST containing 25% methanol
 - Three times with PBST
31. Replace the PBST with the 75% glycerol solution and store the embryos at 4°C until ready for photographing.

When excited with a 633-nm laser, the NBT/BCIP precipitate fluoresces intensely (wavelengths of emitted light are 650 nm) and will quench autofluorescence in lamprey tissues. These properties make it possible to obtain single-cell resolution in whole-mount in situ hybridization and permit multichannel combination of in situ staining with other visible fluorophores (Trinh et al. 2007).

Protocol 5

Immunostaining of Whole-mount and Sectioned Lamprey Embryos

This protocol describes how to immunostain whole-mount or sectioned lamprey embryos using an antibody raised against the protein of interest and detected with an HRP-conjugated secondary antibody. Enzyme-conjugated rather than fluorochrome-conjugated secondary antibodies are used for antigen detection in lamprey embryos because, after fixation, yolk platelet-rich lamprey embryos exhibit a strong autofluorescence in all three channels. Some lamprey antigens (e.g., activated caspase-3 and phospho-histone H3) are recognized by commercially available antibodies raised against antigens from other species.

MATERIALS

The recipes for the items marked with <R> begin on page 426.

CAUTION: See the Cautions Appendix for appropriate handling of materials marked with <!>.

Reagents

3,3-Diaminobenzidine tetrahydrochloride (DAB) <!>

Prepare a stock solution of 2 mg/ml DAB in H₂O, filter it through a 0.22- μ m membrane, and store it at -20°C, protected from light. Before the procedure, dilute the DAB solution to 1:20 in PBST for whole-mount embryos or 1:40 for sections. Staining can be enhanced to give a black signal, which is most useful for cell counting, by adding CoCl₂ <!> and NiCl₂ <!> to final concentrations of 0.1%.

Donkey serum, heat inactivated (5% or 10% [see Step 6], prepared in PBST)

Eukitt <!> (for embryo sections only)

Gelatin (20%), prewarmed for 1 hour at 37°C before use (for embryo sections only)

Glycerol (25, 50, and 75%; prepared in PBST) (for whole-mount embryos only)

Goat-antirabbit antibody, HRP conjugated (1:500, prepared in PBST)

H₂O, distilled (for embryo sections only)

Hydrogen peroxide (H₂O₂; 0.5%, prepared in PBST) <!>

Hydrogen peroxide (30%) <!>

Lamprey embryos, cultured to the desired stage (see Protocol 1)

Liquid nitrogen <!> (for embryo sections only)

MEMFA (for whole-mount embryos only) <R>

Methanol <!> (for whole-mount embryos only)

Paraformaldehyde (4%) <!>

10x PBS <R>, diluted to 1x before use (for embryo sections only)

PBST <R>

PBST-DEPC <R> (for whole-mount embryos only)

In addition, prepare three separate solutions of PBST-DEPC containing 25, 50, and 75% methanol.

Rabbit or mouse antibody, raised against protein of interest (1:250, prepared in PBST)

The required dilution will vary for different antibodies. The stated 1:250 dilution is suitable for rabbit anti-activated caspase-3 (Promega) and should be used as a guide only.

Sucrose (5% and 15%) (for embryo sections only)

Triton X-100 <!> (for embryo sections only)

Equipment

- Centrifuge tubes (15 ml)
- Coplin jars or plastic slide holders (for embryo sections only)
- Cryostat (for embryo sections only)
- Dissection needle (for embryo sections only)
- Microcentrifuge tubes (2 ml; for whole-mount embryos only)
- Molds, rubber (for embryo sections only)
 - Use rubber molds rather than plastic molds to prevent them from cracking when frozen.*
- Nutator (for whole-mount embryos only)
- Superfrost plus slides (for embryo sections only)
- Water bath, preset to 42°C (for embryo sections only)

METHOD

Perform all steps at room temperature unless otherwise noted. Agitate whole-mount embryos on the nutator for all incubations. Incubate embryo sections in Coplin jars without agitation.

Gelatin Embedding and Sectioning

1. Cryoprotect the lamprey embryos by incubating them in 5% sucrose for 5–6 hours at room temperature and then 15% sucrose for 5–6 hours at room temperature or overnight at 4°C.
2. Incubate the embryos in prewarmed 20% gelatin for 4–7 hours at 37°C.
3. Embed lamprey embryos in the desired orientation in 20% gelatin as follows:
 - i. Prepare the mold by filling it halfway with 20% gelatin and allow it to stand for 1–2 minutes to create a solid gelatin “cushion.”
 - ii. Place the embryo in liquid 20% gelatin on top of the “cushion” and adjust the position of the embryo quickly using a hot dissection needle. Allow the gelatin to set for 5–10 minutes.
 - iii. Immerse the entire mold into liquid nitrogen.
 - iv. Store the blocks at –80°C.
4. Make 10–14- μm sections of the blocks using a cryostat and mount the sections on Superfrost plus slides.
5. Store the slides at –20°C.
 - When the slides are taken out of –20°C storage, allow them to warm to room temperature before using.*

Fixation and Blocking

6. Prepare whole-mount embryos or embryo sections for immunostaining as follows.

To Prepare Whole-mount Embryos

- i. Transfer embryos into 2-ml microcentrifuge tubes (up to 20 embryos per tube). Fix the embryos in MEMFA for 1 hour.
- ii. Wash the embryos in PBST-DEPC for 15 minutes. Discard the solution. Repeat this step three more times.

- iii. Wash the embryos for 15 minutes in each of the following four solutions:
 - PBST-DEPC containing 25% methanol
 - PBST-DEPC containing 50% methanol
 - PBST-DEPC containing 75% methanol
 - 100% methanol
- iv. Rinse the embryos several times in methanol.

At this point, embryos can be stored for up to several years at -20°C .
- v. To block endogenous peroxidase activity, treat the embryos with 0.5% H_2O_2 in PBST for 1 hour at room temperature.
- vi. Wash the embryos in PBST three times for 15 minutes each.
- vii. To block nonspecific proteins, incubate the embryos in 10% donkey serum in PBST for 1 hour at room temperature.

To Prepare Embryo Sections

- i. Incubate the slides containing sectioned embryos in preheated (42°C) 1x PBS for 10 minutes in the 42°C water bath to remove the gelatin.
- ii. Wash once for 10 minutes in 1x PBS and then twice for 10 minutes each in PBST.
- iii. To block endogenous peroxidase activity, treat the slides with 0.5% H_2O_2 in PBST for 30 minutes at room temperature.
- iv. Permeabilize the cells by incubating the slides in 0.2% Triton X-100 for 5 minutes then rinse three times in 1x PBS.
- v. Incubate the embryos in 5% donkey serum in PBST for 1 hour at room temperature.

Antibody Binding

7. Incubate the embryos or sections in the antibody raised to the protein of interest overnight at 4°C .
8. Wash the antibody from the samples as follows:
 - For whole-mount embryos, wash five to eight times with PBST for 1 hour each. Then incubate overnight in PBST at 4°C .
 - For sections, wash twice with PBS for 10 minutes each. Then wash twice with PBST for 10 minutes each.
9. Incubate the samples in HRP-conjugated goat-antirabbit antibody for 4 hours (for whole-mount embryos) or 2 hours (for sections) at room temperature.
10. Remove unbound antibodies as follows:
 - For whole-mount embryos, rinse five times in PBST. Then wash overnight in PBST at 4°C .
 - For sections, wash twice in PBS for 10 minutes each. Then wash twice with PBST for 10 minutes each.

Color Development

11. Add 10 ml of DAB solution to the embryos in a 15-ml plastic centrifuge tube.

If using embryo sections, skip Step 12 and proceed to Step 13 to allow the DAB to penetrate.
12. For whole-mount embryos, incubate for 30 minutes at room temperature.

13. Add 3.3 μ l of 30% hydrogen peroxide and incubate for 30–45 minutes, checking regularly for color development.
If the color has not yet developed, the DAB/hydrogen peroxide mix can be replaced after 30 minutes.
14. When sufficient color formation has occurred, stop the reaction by washing whole-mount embryos in several changes of PBST or by dipping embryo section slides several times in a 1-liter beaker containing distilled H₂O.
15. Postfix whole-mount embryos and sections in 4% paraformaldehyde for 20–30 minutes at room temperature.
16. Prepare the stained samples for photographing.

To Prepare Stained Whole-mount Embryos

- i. Rinse three–four times for 5 minutes each in PBST
- ii. Process through glycerol/PBST series: 25%, 50%, 75%.
- iii. Photograph in 75% glycerol.

Whole-mount embryos can be processed to gelatin and sectioned as described on page 422.

To Prepare Stained Embryo Sections

- i. Dehydrate the slides.
- ii. Mount slides in Eukitt.
- iii. View and photograph.

Recipes

The recipes for items marked with <R> are also listed here.

CAUTION: See the Cautions Appendix for appropriate handling of materials marked with <!>.

BLEACHING SOLUTION

Reagent	Quantity (for 10 ml)	Final concentration
Formamide <!>	500 μ l	5%
H ₂ O ₂ <!>	2.8 ml	28%
20x SSC	250 μ l	0.5x
H ₂ O, DEPC-treated	to 10 ml	

Prepare fresh. To avoid foaming, add the ingredients in the following sequence: formamide, DEPC-treated H₂O, 20x SSC, and H₂O₂. After use, dilute the bleaching solution with an equal amount of H₂O and store it in the fume hood before disposal.

BLOCKING SOLUTION

Reagent	Quantity (for 100 ml)	Final concentration
Blocking reagent, 10% stock solution in 1x MAB (Roche)	20 ml	2%
Sheep serum, heat inactivated	20 ml	20%
MABT <R>	to 100 ml	

This solution can be stored frozen for several months at -20°C.

COLOR DEVELOPMENT SOLUTION

Reagent	Quantity (for 10 ml)	Final concentration
4-Nitro blue tetrazolium chloride (NBT) <!> (100 mg/ml)	45 μ l	0.45 mg/ml
5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) <!> (50 mg/ml)	35 μ l	0.175 mg/ml
NTMT <R>	to 10 ml	

Prepare just enough color development solution for the reaction. Excess can be stored for several days in foil-covered tubes at 4°C.

HYBRIDIZATION MIX

Reagent	Quantity (for 500 ml)	Final concentration
CHAPS <!-- (10% in DEPC-treated H ₂ O) -->	25 ml	0.5%
EDTA (0.5 M, pH 8.0) <!-- -->	5 ml	5 mM
Formamide, deionized <!-- (Ambion) -->	250 ml	50%
Heparin <!-- -->	50 mg	100 µg/ml
20x SSC	32.5 ml	1.3x
tRNA from baker's yeast	100 mg	200 µg/ml
Tween 20	1 ml	0.2%
H ₂ O	to 500 ml	

Hybridization mix can be stored for several months at -20°C.

1x MABT

Reagent	Quantity (for 1000 ml)	Final concentration
Maleic acid <!-- -->	11.6 g	0.1 M
NaCl	8.77 g	150 mM
Tris base <!-- -->	20–30 g	0.2 M
Tween 20	1 ml	0.1%
H ₂ O	to 1000 ml	

Adjust pH to 7.5 with Tris base. Filtered MABT can be stored for a few weeks to several months.

MEMFA

Reagent	Quantity (for 10 ml)	Final concentration
Formaldehyde, ultrapure <!-- (16%) -->	2.5 ml	4%
10x MEM salts <R-->	1 ml	1x
H ₂ O, distilled	to 10 ml	

Store for 5–12 hours at 4°C, protected from light.

10x MEM SALTS

Reagent	Quantity (for 500 ml)	Final concentration
MOPS (pH 7.4) <!-- -->	104.65 g	1 M
EGTA	3.804 g	20 mM
MgSO ₄ <!-- -->	0.602 g	10 mM
H ₂ O, distilled	to 500 ml	

This solution can be stored for several months at room temperature, protected from light.

10x MMR

Reagent	Quantity (for 1000 ml)	Final concentration
CaCl ₂ <!\> • 2H ₂ O	2.94 g	20 mM
EDTA (0.5 M) <!\>	2 ml	1 mM
HEPES (1 M)	50 ml	50 mM
KCl <!\>	1.491 g	20 mM
MgSO ₄ <!\>	1.204 g	10 mM
NaCl	58.44 g	1 M
H ₂ O	to 1 liter	

Mix all ingredients well before adding CaCl₂. Adjust pH to 7.8 with 1 M NaOH. MMR is prepared as a 10x stock, then diluted for 1x or 0.1x with distilled H₂O, and autoclaved before use. The 10x stock can be stored for several months to years at room temperature.

NTMT

Reagent	Quantity (for 200 ml)	Final concentration
MgCl ₂ (2 M)	5 ml	50 mM
NaCl (5 M)	4 ml	100 mM
Tris-Cl (1 M, pH 9.5)	20 ml	100 mM
Tween 20	200 µl	0.1%
H ₂ O	to 200 ml	

There is no need to adjust pH if stock Tris-HCl solution is used. This solution should be prepared fresh before use. It can be stored for 12–24 hours at room temperature. Precipitates form when stored for longer periods.

10x PBS

Reagent	Quantity (for 1 liter)	Final concentration
NaH ₂ PO ₄	2.75 g	23 mM
NaCl	90 g	1.54 M
Na ₂ HPO ₄ • 7H ₂ O	21.45 g	80 mM
H ₂ O	to 1 liter	

Adjust pH to 7.4 with NaOH <!\>. Store for several months at room temperature.

PBST

Reagent	Quantity (for 1 liter)	Final concentration
10x PBS <R>	100 ml	1x
Tween 20	1 ml	0.1%
H ₂ O	to 1 liter	

Store at room temperature.

PBST-DEPC

Reagent	Quantity (for 1 liter)	Final concentration
10x PBS <R>	100 ml	0.1x
Diethylpyrocarbonate (DEPC) <R>	1 ml	0.1%
Tween 20	1 ml	0.1%
Distilled H ₂ O	to 1 liter	

Stir at room temperature for 1 hour and then autoclave for 1 hour to remove the DEPC. Add Tween 20 after autoclaving. Store for months to years at room temperature.

POSTFIX SOLUTION

Reagent	Quantity (for 10 ml)	Final concentration
Glutaraldehyde <R> (25%)	80 µl	0.2%
Paraformaldehyde <R>	400 mg	4%
PBST-DEPC <R>	to 10 ml	

Prepare solution just before use. Do not store for more than 1–2 hours at 4°C.

REFERENCES

- Alder, M.N., Rogozin, I.B., Iyer, L.M., Glazko, G.V., Cooper, M.D., and Pancer, Z. 2005. Diversity and function of adaptive immune receptors in a jawless vertebrate. *Science* **310**: 1970–1973.
- Amemiya, C.T., Saha, N.R., and Zapata, A. 2007. Evolution and development of immunological structures in the lamprey. *Curr. Opin. Immunol.* **19**: 535–541.
- Cooper, M.D. and Alder, M.N. 2006. The evolution of adaptive immune systems. *Cell* **124**: 815–822.
- Damas, H. 1944. Recherches sur le developpement de *Lampetra fluviatilis* L. Contribution a l'etude de la cephalogenese des vertebres. *Arch. Biol.* **55**: 3–282.
- Gill, H.S., Renaud, C.B., Chapleau, F., Mayden, R.L., and Potter, I.C. 2003. Phylogeny of living parasitic lampreys (Petromyzontiformes) based on morphological data. *Copeia* **2003**: 687–703.
- Hardisty, M.W. 1979. *Biology of the cyclostomes*. Chapman and Hall, London.
- Hardisty, M.W. and Potter, I.C., eds. 1971a. Paired species. In *The biology of lampreys* (ed. M.W. Hardisty and I.C. Potter), pp. 249–278. Academic, London.
- Hardisty, M.W. and Potter, I.C., eds. 1971b. The general biology of adult lampreys. In *The biology of lampreys* (ed. M.W. Hardisty and I.C. Potter), pp. 127–206. Academic, London.
- Hardisty, M.W. and Potter, I.C., eds. 1971–1982. *The biology of lampreys*. Academic, London.
- Horigome, N., Myojin, M., Ueki, T., Hirano, S., Aizawa, S., and Kuratani, S. 1999. Development of cephalic neural crest cells in embryos of *Lampetra japonica*, with special reference to the evolution of the jaw. *Dev. Biol.* **207**: 287–308.
- Hubbs, C.L. and Potter, I.C. 1971. Distribution, phylogeny and taxonomy. In *The biology of lampreys* (ed. M.W. Hardisty and I.C. Potter), vol. 1, pp. 1–65. Academic, London.
- Huxley, T.H. 1876. The nature of the craniofacial apparatus of *Petromyzon*. *J. Anat. Physiol.* **10**: 412–429.
- Kuratani, S., Kuraku, S., and Murakami, Y. 2002. Lamprey as an evo-devo model: Lessons from comparative embryology and molecular phylogenetics. *Genesis* **34**: 175–183.
- McCauley, D.W. and Bronner-Fraser, M. 2003. Neural crest contributions to the lamprey head. *Development* **130**: 2317–2327.
- McCauley, D.W. and Bronner-Fraser, M. 2006. Importance of SoxE in neural crest development and the evolution of the pharynx. *Nature* **441**: 750–752.
- Nagawa, F., Kishishita, N., Shimizu, K., Hirose, S., Miyoshi, M., Nezu, J., Nishimura, T., Nishizumi, H., Takahashi, Y., Hashimoto, S., et al. 2007. Antigen-receptor genes of the agnathan lamprey are assembled by a process involving copy choice. *Nat. Immunol.* **8**: 206–213.
- Osorio, J. and Retaux, S. 2008. The lamprey in evolutionary studies. *Dev. Genes Evol.* **218**: 221–235.
- Pancer, Z., Amemiya, C.T., Ehrhardt, G.R., Ceitlin, J., Gartland, G.L., and Cooper, M.D. 2004. Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* **430**: 174–180.
- Piavis, G.W. 1961. Embryological stages in the sea lamprey and effect of temperature on development. *U.S. Fish Wildl. Serv. Fish. Bull.* **61**: 111–143.
- Piavis, G.W. 1971. Embryology. In *The biology of lampreys* (ed. M.W. Hardisty and I.C. Potter), vol. 1, pp. 361–400. Academic, London.
- Reese, A.M. 1900. Lampreys in captivity. *Science* **11**: 555.
- Rubinson, K. 1990. The developing visual system and metamorphosis in the lamprey. *J. Neurobiol.* **21**: 1123–1135.
- Sauka-Spengler, T., Meulemans, D., Jones, M., and Bronner-Fraser, M. 2007. Ancient evolutionary origin of the neural crest gene regulatory network. *Dev. Cell* **13**: 405–420.
- Stock, D.W. and Whitt, G.S. 1992. Evidence from 18S ribosomal RNA sequences that lampreys and hagfishes form a natural group. *Science* **257**: 787–789.
- Tahara, Y. 1988. Normal stages of development in the lamprey *Lampetra reissneri* (Dybowski). *Zool. Sci.* **5**: 109–118.
- Takezaki, N., Figueroa, F., Zaleska-Rutczynska, Z., and Klein, J. 2003. Molecular phylogeny of early vertebrates: Monophyly of the agnathans as revealed by sequences of 35 genes. *Mol. Biol. Evol.* **20**: 287–292.

Trinh, L. A., McCutchen, M.D., Bonner-Fraser, S.E., Bumm, L.A., and McCauley, D.W. 2007. Fluorescent in situ hybridization employing the conventional NBT/BCIP chromogenic stain. *Biotechniques* **42**: 756–759.

Vladykov, V.D. 1949. Quebec lampreys (Petromyzonidae). Department of Fisheries, Province of Quebec, Canada. Contribution 26, pp. 1–6.

WWW RESOURCES

<http://www.invasivespeciesinfo.gov/aquatics/lamprey.shtml> The National Invasive Species Information Center. This website has

numerous links to and information about *P. marinus*, its biology, and efforts to control its impact on native species.