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Inferring developmental constraint and constraint release: Primordial germ cell determination mechanisms as examples

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Abstract

Developmental constraint and its converse constraint release are significant concepts in understanding pattern and process in macroevolution. The purpose of this paper is to propose a two-step method for identifying constraints and constraint release. The first step is a phylogenetic optimization procedure to identify which trait/process is primitive and which is derived. The primitive trait is inferred to be the constraint and the convergently derived trait the release. The second criterion uses sister-clade asymmetry. Clades diagnosed by the constraint will have fewer taxa than clades diagnosed by the release. As an example, we use the process of germ cell specification, in which there are three modes of specification. Our results corroborate previous conclusions that the induced mode is the constraint and the predetermined mode is the release and we speculate on the importance of these two processes in terms of robustness and evolvability.

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"In effect, virtually every developmental process has been cited, at one time or another, as a possible developmental constraint, as has every conceivable property of developmental systems, from the genetic to the physical to the mathematical." (Wilkins, 2002, p. 385).

1. Introduction

Central to the evolution and development research program is the concept of developmental constraint and its converse, constraint release. What we call constraint release is basically an evolvability issue, at least as that term has been recently used (Arnold, 1989; Dawkins, 1989; Kirschner and Gerhart, 1998; Kitano, 2004; Schlichting and Murren, 2004; Wagner, 2005). Evolvability can be generalized as the ability or capacity of an organism or lineage to produce novel phenotypic variation (Schlichting and Murren, 2004). Evolvability can also be considered the converse of rigidity in genetic programming, implying the existence of loosened developmental and genetic programs. The consequence of such loosened systems may, as described by Dawkins (1989), open the "floodgates to future evolution." For this reason, we see evolvability as an issue of constraint release, which is the escape from the conserved, constrained program.

Identification of a developmental constraint is recognized as a challenging proposition for a number of reasons, beginning with debate on the definition of such a constraint. As a general biological concept, constraint is the inhibition of change to proceed beyond a limit or boundary. These constraints can be physical or physiological or developmental. For example, terrestrial organisms with exoskeletons cannot achieve large size because of the physical constraint imposed by scaling. Mammals cannot be the size of small insects because of physiological limits. The gas exchange system is too inefficient at that size and cannot supply enough oxygen. In development, probably every conserved mechanism has been considered a

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constraint. Examples might be the tinman/NKX2-5 gene expression for heart development (Bodmer, 1993; Manak and Scott, 1994), or the expression of PAX 6 for sensory organs, especially eye development (Halder et al., 1995; Chow et al., 1999; Onuma et al., 2002).

Developmental constraint as a rigid limit or boundary had long ago been softened to a strong bias (e.g. Alberch, 1980; Maynard Smith et al., 1985) and not thought of as an all or nothing proposition. A mere bias implies the presence of variation in developmental processes (and thus outcomes). It followed that with the recognition of variation natural selection must play a key role. The idea of constraint as internal selection was a natural extension of the implied developmental variation (Arthur, 1997; Wagner and Schwenk, 2000). While variation may indeed exist in development, the perception is that it is absent because selection inhibits successful development of variable outcomes. An interesting flip side to the relationship between selection and constraint is that a constraint could be something that limits the ability of natural selection to change development. Regardless, a developmental constraint can be summarized as a feature(s) of development that inhibits certain pathways or change or that narrows evolutionary options.

We agree with Schwenk and Wagner and see constraint/constraint release as relative concepts; therefore, any discussion of constraint must be made in specific relation to something else (Schwenk and Wagner, 2003, 2004). Following the idea of constraint as a relativistic concept, if constraint is some sort of bias or inhibition, then developmental constraint *release* would be an escape from the constraint and only recognizable in comparison to the hypothesized constraint. Both constraint and constraint release should be identifiable based on certain criteria; we suggest such criteria below. These are not necessarily mutually exclusive criteria, nor necessarily universal, but when correlated strengthen the discovery claims of constraint and constraint release.

Because our goal is to operationalize the relativistic concept, our criteria, importantly, are relative concepts themselves: synapomorphy and clade asymmetry. We adopt Schwenk and Wagner's concept and employ relative concepts in our operationalization of it because we view any macroevolutionary research program as necessarily comparative. Simply, apomorphy cannot be identified in the absence of plesiomorphy and in the same way, constraint cannot be identified in the absence of release. Without comparison, how can it be determined that all the traits are apomorphies or plesiomorphies, or all constraints or all releases? The same is true for clade asymmetry. Is a clade of 50 species large? That question cannot be answered until the clade is compared with its sister clade. Constraint may indeed exist without relativism, but we could not recognize it.

We propose two criteria to identify constraint and release, and both criteria require a phylogenetic framework (see Richardson et al., 2001). A trait (e.g. developmental

process) that has a phylogenetic distribution that appears rare (at the particular level of investigation) and convergent is considered evidence for constraint release (following the idea of Sommer, 1999). Its converse, a primitive trait, can be inferred to be a constraint (Richardson et al., 2001; Fig. 1A). By definition and logical extension, a constraint would be expected to be common (i.e. unchanged) across clades whereas release from that constraint would be expected to appear intermittently across clades. This operational approach to the identification of primitive and derived traits follows the standard methodology as described by Hennig (1966) and modified and used in myriad ways since then (e.g. Maddison and Maddison, 2000; Brooks and McLennan, 1992, 2002).

Maynard Smith et al. (1985) and Raff (1996) argued for the same criterion but from a different perspective; an independently evolved variant in different lineages indicates the presence of a common constraint. For example, if variant A evolves in unrelated clades, it may indicate an escape from a constraint shared across those clades. Whether or not the constraint is phylogenetically common because it is developmentally difficult to overcome or because genetic variation in the constraint is lacking is not an issue because both still result in constraints being phylogenetically more common than escapes from the constraint (Wagner and Müller, 2002).

Given our acceptance of constraint as a bias in development, why not consider independently evolved processes or structures with similar phenotypes as evidence
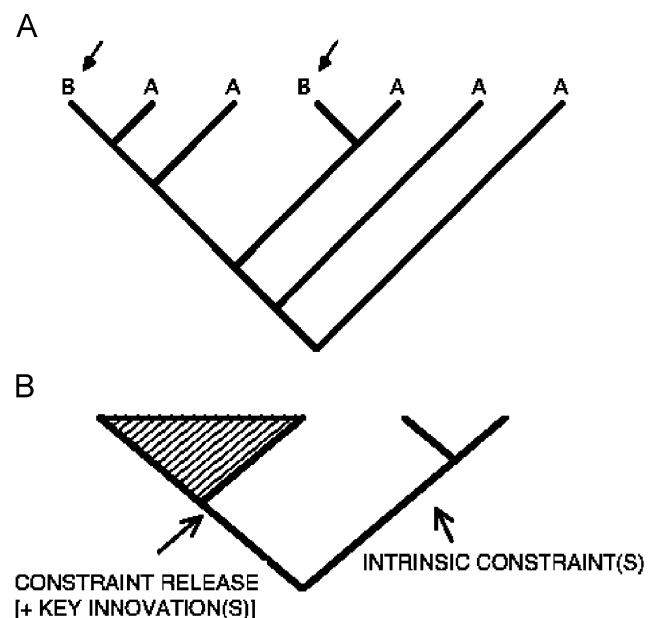

Fig. 1. Illustrations of the proposed criteria for identifying developmental constraint and constraint release. (A) Distribution of characters A and B, where A is basal and primitive and B is convergent and derived. Character A is interpreted as a constraint and character B as a release. (B) Cartoon of an unbalanced tree with asymmetrical sister clades. The depauperate clade indicates the presence of a constraint whereas the speciose clade indicates the release from the constraint that allows the development of key innovations. See text for further discussion.

for constraints? We disagree with that interpretation for two reasons. One, independent evolution of the same process or similar phenotype in unrelated lineages signals an escape route from the constraint. Perhaps in the evolution of future lineages of the release, the release, in comparison to new traits in new lineages, will be viewed as a constraint. But because we see constraint as relativistic, at the moment of comparison the common primitive trait, the one found across lineages, the conserved trait, is considered the constraint. That is the second reason. Constraint equals conservation of the trait. Release is evolution away from the conserved trait.

The second criterion, one that strengthens the claims of the first, is the presence of asymmetrical sister clades. The use of sister group analysis in comparative biology is recognized as a powerful method (e.g. Harvey and Pagel, 1991; Brooks and McLennan; 1992, 2002; Barraclough et al., 1998) for identifying features that are correlated with differential speciation rates. Barraclough et al. (1998, p. 751) are clear and convincing in arguing that "sister-group comparisons are the most statistically powerful approach for identifying correlates for net diversification rates".

Although this may not always be the case, inhibition of phenotype evolution via constraint could also inhibit high species diversity, i.e. evolvability, as a consequence of reduced rates of speciation. Conversely, the release of that constraint should provide opportunity for morphological reorganization, essentially experimentation during morphogenesis, and lead to the production of novel phenotypes that may include key innovations, increased rates of speciation and consequently clade diversification. Some

key innovation(s) would be required before adaptive radiation would occur (e.g. Erwin and Karakauer, 2004). So we might expect examination of sister clades with different developmental processes (one primitive and the other derived based on the first criterion) to yield sister clades of significantly different sizes in terms of species number. The constrained clade would have relatively few taxa compared to the released clade (Fig. 1B).

The potential exists for key innovation and release to be confused. Release and innovations are independent concepts and occur at different levels of the event cascade (Fig. 2). Release must always occur first. Release provides the opportunity for development of novel phenotypes, and included among these novel phenotypes may be a key innovation(s). The identification of clades released from constraint points to places to search for key innovations. Our criteria identify constraints and constraint releases, not the innovations that can or have arisen by virtue of the release.

We use the specification of primordial germ cells (PGCs) as an example of this two criteria approach to identify developmental constraint and release. One mode of PGC specification requires inducing signals to form a differentiated germ line late in development. This mode is referred to as regulative, induced, or epigenetic. In an alternative mode the PGCs are set aside early, and specified by maternally deposited germ cell determinants. This is designated the preformation or predetermined mode. A third mode of PGC specification, somatic embryogenesis (Buss, 1983), is present in basal metazoan lineages (pre-bilateral organization) and in a few more derived clades, In somatic embryogenesis, there is no differentiated germ line;
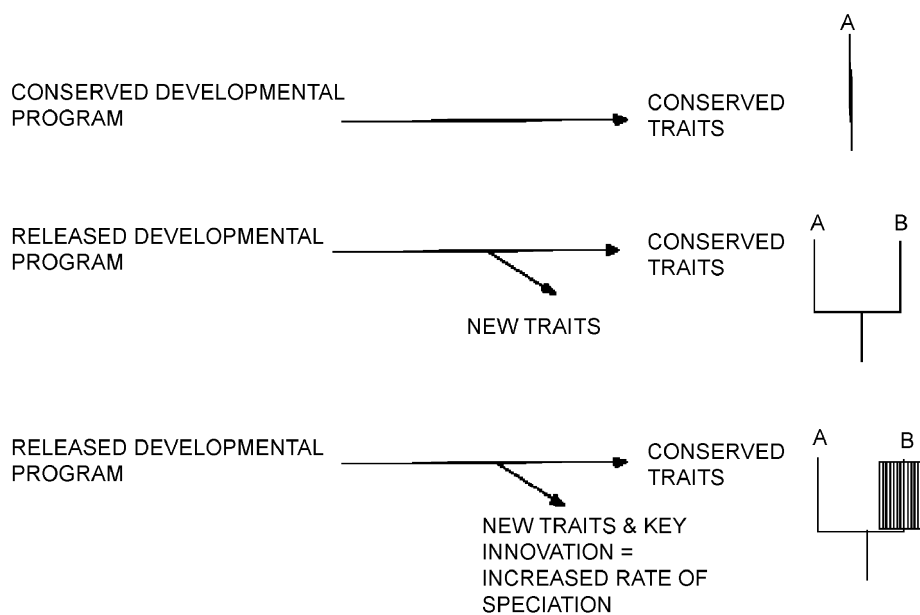

Fig. 2. Hypothesis of the relationships between constraint release, key innovations, and rate of speciation. In the simplest model, conserved developmental programs do not produce variable phenotypes and speciation is minimized, shown here as a single lineage A. As development moves away from the bias, or constraint, novel traits (apomorphies) can be produced, leading to speciation. B represents a new lineage diagnosable by the new traits. In the third diagram, expanded release of the constraint results in the production of many new traits, some of which function as key innovations. The accumulation of key innovations and consequently apomorphies result in increased rates of speciation and yield asymmetric sister clades.

instead, germ cells are developed from somatic cells throughout the life of the organism. Unlike the vast majority of developmental mechanisms, which appear to be conserved during evolution, the modes of PGC specification are clearly not conserved. Indeed the distinct modes of PGC specification show a curious distribution throughout divergent lineages. Given the obvious importance of PGC development to the maintenance of an organism's lineage, we regard germ cell determining mechanisms as an ideal character to test our two-step model for identifying developmental constraints.

2. PGC determination mechanisms

Among most bilateral animals there exist two very different means for specifying PGCs during development. In the embryos of some species, PGCs are specified cell-autonomously by maternal molecules known collectively as germ plasm. PGCs formed in this way are derived from *predetermined* (sometimes referred to as preformed) precursors that are destined to enter the germ line from the inception of development. Germ plasm is typically localized to a discrete region of the egg cytoplasm and is then differentially distributed to presumptive germ cells during embryogenesis. Germ plasm can be identified at the ultrastructural level as a region of densely packed mitochondria, endoplasmic reticulum, and electron dense structures known as germinal granules. Moreover, work in recent years shows that germ plasm also contains messenger RNAs encoding germ cell-specific RNA binding proteins that are thought to mediate the process of germ line segregation (Houston and King, 2000; Hashimoto et al., 2004). Evidence from diverse species suggests that germ plasm acts by blocking the transcriptional and/or translational response to extracellular signals that might otherwise divert the presumptive germ cells toward a somatic cell fate (Leatherman and Jongens, 2003; Blackwell, 2004), terminating the germ line. Predetermined germ cells are therefore thought to develop independent of the zygotic influences that govern development of the soma.

Other species rely on extracellular signals to *induce* PGCs from pluripotent precursor cells [e.g. mouse (Lawson and Hage, 1994); axolotl (Bachvarova et al., 2004)]. In these organisms, PGCs are specified by zygotic, not maternal, influences, and this is sometimes referred to as an epigenetic or regulative mode. Importantly, in this mode PGCs are derived from unspecialized cells that can also contribute to somatic lineages; the specification of PGCs is therefore governed by a precise complement of extracellular signals secreted by surrounding somatic tissue. Thus regulative PGC specification is dependent on an interaction between germ line and soma that is not required in embryos with predetermined germ cells.

Somatic embryogenesis, or multipotency, can be considered the most plastic of the three modes of PGC determination. No germ line is dedicated prezygote and no cell lineage is specifically induced to form the germ line at a specific time later in embryogenesis. In contrast, somatic embryogenesis does not constrain development of germs cells to a specific period of development, but is a process that produces germ cells throughout the individual's ontogeny.

The mechanism of germ cell determination has only been definitively determined in a relatively small number of animals. Classical embryological model systems including the nematode *C. elegans*, the fruit fly *Drosophila*, and the frog *Xenopus laevis* have been shown to experimentally contain germ plasm, and for many years predetermination was considered to be the typical state. More recently zebrafish embryos have been shown to contain localized germ cell determinants as well (Hashimoto et al., 2004). However, Johnson et al. (2001) showed that embryos from the axolotl, a caudate amphibian, do not have germ plasm, and although Nieuwkoop and others suggested as early as 1969 that salamander PGCs could be induced (Nieuwkoop, 1969; Sutasurya and Nieuwkoop, 1974; Michael, 1984; Maufroid and Capuron, 1985), these and similar results were largely ignored in the general germ cell literature. It was not until several years after Tam and Zhou (1996) conclusively showed regulative specification of mouse PGCs that the distribution of regulative and predetermined germ cell determining mechanisms in divergent animal lineages became widely acknowledged. Ransick et al. (1996) demonstrated experimentally that PGCs in sea urchins are not exclusively dependent on germ plasm, suggesting that regulative germ specification might be basal to the deuterostomes. So even though direct evidence for PGC determination mechanism remains confined to a few model systems, the evidence suggests that the evolutionary history of these mechanisms is more complicated than previously thought.

3. Criterion 1: primitive or derived?

The three PGC determination mechanisms were treated as separate character states and fit to a generally accepted (though details are debated) phylogeny of metazoans. The phylogeny is a combination of trees from recent reviews on invertebrates (Halanych, 2004) and vertebrates (Meyer and Zardoya, 2003). The PGC determination modes for each taxon were based on data from Blackstone and Jasker (2003), Extavour and Akam (2003), and Johnson et al. (2003a, b). Blackstone and Jasker (2003) provided the data for the somatic embryogenesis state. The states were optimized onto the tree using the program MacClade (Maddison and Maddison, 2000), in which all the most parsimonious reconstructions were examined for determining the ancestral conditions. Regardless of the optimization procedure, the character states' fit indicate that relative to one another, the induced mode is primitive and the predetermined mode is derived (Fig. 3) in the Bilateria (the bilateral animals, from the Acoelomorpha and above). For the Metazoa, somatic embryogenesis appears primitive, but within the Bilateria, the induced mode is primitive and
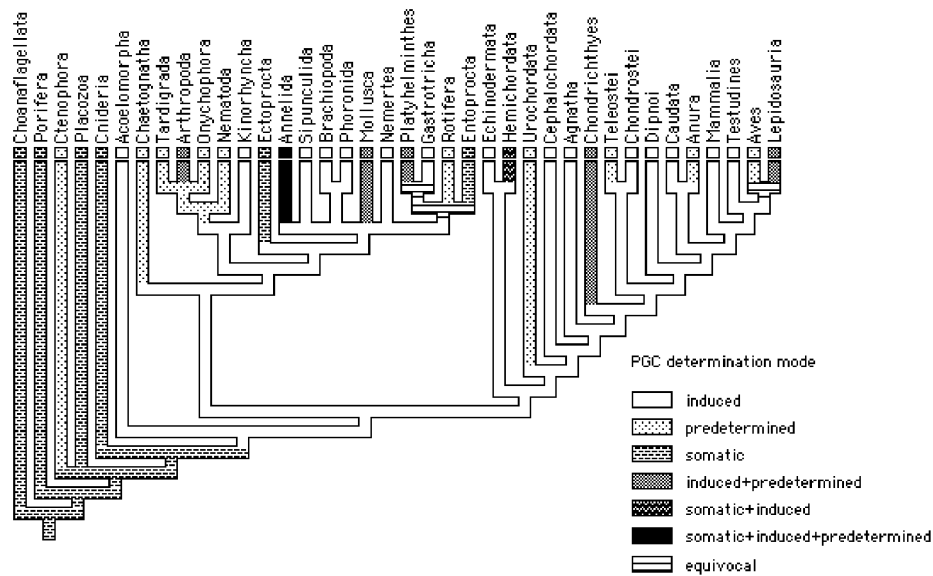
Fig. 3. Distribution of PGC determination mechanisms among metazoans. The evolution of germ cell determination mode was inferred by parsimony optimization in MacClade (v. 4.0, Maddison and Maddison, 2000). See the legend in the figure for character types. Some clades are presumed to exhibit both modes (e.g. in the Lepidosauria, snakes are presumed to have the predetermined mode whereas iguanids have the induced mode (Hubert, 1985) and are shown here. Branch lengths are arbitrary and not intended to indicate evolutionary distance. See the text for further explanation.

somatic embryogenesis apparently re-evolved four times. The method and interpretation of results are based on the standard definition of primitive and derived characters when examined on a phylogeny (e.g. Hennig, 1966). Our conclusion that the induced mode is primitive within bilateral animals is no longer surprising and had been previously hypothesized by Blackstone and Jasker (2003), Extavour and Akam (2003), and Johnson et al. (2003a, b).

Although the predetermined mode has long been considered the default (i.e. primitive) for bilateral animals, our results reject that hypothesis and corroborate the alternative which suggests the predetermined mechanism seen in frogs and zebrafish is convergently derived (Johnson et al., 2003a, b). A recent review stated that while *model* organisms tend to show the preformed (predetermined) mode, the epigenetic (induced) mode is actually more common (Extavour and Akam, 2003). Extavour and Akam further suggested that the induced mode is probably primitive among metazoans but they did not differentiate the somatic embryogenesis mode. Our analysis indicates that for metazoans somatic embryogenesis is primitive, but for bilaterians, the induced mode is primitive and the predetermined mode is derived. Therefore, based on the first criterion for identification of a constraint as primitive and constraint release as convergently derived, the induced mode is the constraint and the predetermined mode is the constraint release.

4. Criterion 2: asymmetrical sister clades

The second criterion says that when comparing sister clades with different developmental mechanisms, if one is a constraint and the other is the release, clade size asymmetry

is possible. The constrained clade would be depauperate and the released clade would be speciose relative to its sister. We compared pairs of sister clades, with each clade containing a different mode of PGC determination. Among the Amphibia, Caudata, with the primitive induced mode, has approximately 415 species and its sister clade with the predetermined mode, the Anura, has approximately 4300 species (Pough et al., 2004). Among fishes, the basal actinopterygian (although this is a paraphyletic group, we include them all to increase species numbers) species count is 44 and the sister clade, the teleosts, have approximately 17,000 species (Nelson, 1994). Evidence from Johnson (unpublished data) suggests that actinopterygian oocytes do not contain localized germ cell determinants. Extavour and Akam (2003) presented evidence that indicates that the basal lineages of the Mollusca use the induced mechanism. They also suggest the crown clades, Cephalopoda, Bivalvia, and Gastropoda, use the predetermined mode (no data on Scaphopoda; it may be more complicated among gastropods, there is some evidence for the induced mode as well, Extavour and Akam, 2003, Table 2, p. 5876). If these modes are correct, the sister clade asymmetry is once again large: basal lineages include 1400 species and crown clades contain about 92,000 species (Brusca and Brusca, 2003) (Fig. 4). This second criterion also points to the induced mode as a developmental constraint and the predetermined mode as a constraint release.

For the comparisons we can make, the question arises, are these asymmetries different from a stochastic expectation? An asymmetrical sister clade relationship has been shown to be as equiprobable as any other numerically divided topology (Farris, 1976; Slowinski and Guyer, 1989; Maddison and Slatkin, 1991). Explanations about key
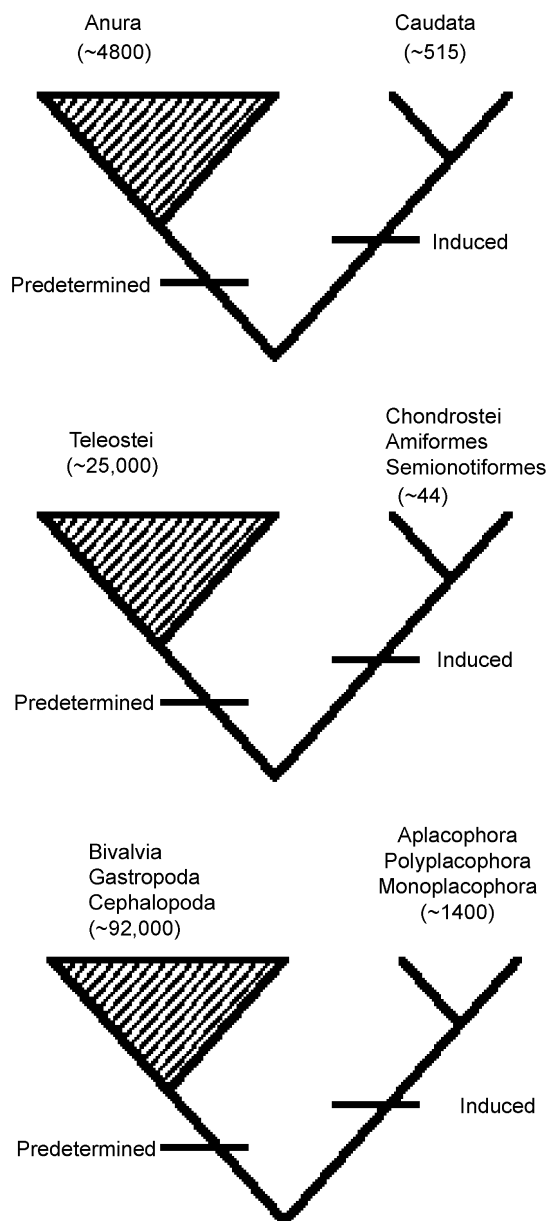
Fig. 4. Relationship between PGC determination mechanism and asymmetrical sister clades. These sister clade comparisons indicate disparate rates of speciation associated with mode of PGC determination. Each comparison shows the induced mode correlated with depauperate clades while the predetermined mode is correlated with speciose clades (1–3 orders of magnitude greater). See text for elaboration.

innovations for speciose clades may by themselves be simply adaptive story telling and "not prima facie evidence that the group arose from non-random speciation and/or extinction" (Guyer and Slowinski, 1993). While tree balance itself can be used to infer something about evolution, when a feature (e.g. a putative innovation or developmental mechanism) is correlated with the tree shape then the inference becomes more powerful and leads toward explanation (e.g. Mooers and Heard, 1997; Barraclough et al., 1998).

Because asymmetrical sister clades can be a stochastic result, various statistical tests have been developed. When

several sister clades possessing the same trait are compared, one can determine the probability that the clade asymmetry is simply a result of chance or infer a cause-and-effect relationship between the trait and asymmetrical clades (Vamosi and Vamosi, 2005). Unfortunately, such sister comparisons are few with the available developmental data and not yet amenable to statistical analysis. A simple bar graph would only show the obvious, all possible sister-group comparisons show asymmetry; therefore, increased rates of speciation are always associated with the pre-determined mode.

It is interesting to note that the opposite hypothesis was proposed by Buss (1983, 1988), He looked at overall species numbers of metazoan groups and found the pattern that phyla with the predetermined mode had lower species numbers than phyla that exhibited the induced modes (either one). The addition of data since then changes the pattern. For example, most of the Mollusca actually exhibit the predetermined mode and only the basal lineages show the induced mode. Also, in chordates, which exhibit all three modes, the two largest clades, teleost fish and birds, also exhibit the predetermined mode.

5. Discussion

5.1. The method

The two-step approach we proposed here has the advantage of requiring multiple lines of evidence for tests of hypotheses of developmental constraint and release. The strongest tests require robust highly corroborated phylogenies that are used as the framework for determining the evolution of the traits/processes in question. Weak phylogenetic frameworks, while providing an opportunity to propose hypotheses of constraint and release, are likely to change and with it reject the constraint hypotheses. This same point is true for the second step, the identification of asymmetrical sister clades. Not only must the phylogenies be well corroborated, but they also must be well resolved. Partially unresolved phylogenies, even if well corroborated, can exhibit apparent asymmetry that exists only as an artifact of the polytomies. The second step adds further rigor to the test by requiring phylogenies from multiple groups of organisms in which each sister clade exhibits a different trait/process. If sister clades possess the same trait yet exhibit asymmetry, we see that as a rejection of a hypothesis of constraint and release. In some cases asymmetry will exist under both circumstances, i.e. with different traits and with the same trait. Adding power to the method, this step is amenable to statistical testing when multiple such comparisons can be made for the traits (Barraclough et al., 1998).

5.2. The biology

The conclusions from our results, the predetermined PGC determination mechanism is derived and a constraint

release and the induced mechanism is primitive and a constraint seems to be at odds with an earlier hypothesis proposed by Buss (1983, 1988). Buss argued that the mechanism found among most basal metazoan lineages, somatic embryogenesis, is primitive and not a constraint, but that the predetermined and induced modes were constraints relative to somatic embryogenesis. Buss developed his arguments based on a comparison of the number of species in phyla and their associated PGC determination mechanism. In the comparison, he noted that phyla with somatic embryogenesis had more species than phyla with only the predetermined mode or the induced mode. Since then, much data on PGC determination has been accumulated across the Metazoa (see Blackstone and Jasker, 2003; Extavour and Akam, 2003). When those data are coupled with a phylogenetic comparative approach in which sister clades are compared, the pattern observed by Buss does not hold up, especially when looked at below the phylum level. Buss may very well be correct in asserting that somatic embryogenesis is unconstrained and provides a novel pathway for transmitting heritable information to subsequent generations. But with sister group comparisons, it is clear that the predetermined mode has significantly more species and is derived. Interestingly, Buss (1988) recognized that the predetermined mode was correlated with the most speciose clades of higher taxa, therefore did not consider the predetermined mode a constraint at that level, which is a prediction of our conclusions,

> All higher taxa which have undergone substantial amplification of a given Bauplan are taxa in which early embryonic germ-line sequestration is not primitive. (Buss, 1988: p. 315).

The evidence we present here indicates that the evolution of predetermined germ cells improves evolvability within specific lineages of animals, presumably through constraint release. Ultimately, increased evolvability is thought to result from an enhancement of an organism's robustness, as defined by an improved capacity to accumulate non-lethal genetic mutations that lead to greater genetic diversity and enhanced speciation (Kirschner and Gerhart, 1998; Kitano, 2004; Wagner, 2005). This raises the question of how the evolution of predetermined germ cells would enhance robustness? In considering how the evolution of biochemical mechanisms can lead to enhanced evolvability, Kirschner and Gerhart (1998) describe the concept of weak linkage, in which the loosening of interactions between molecules leads to a relaxation of constraints, and enhanced robustness. We view the effects of evolution of predetermined germ cells in an analogous way that we relate to embryological events.

The germ line and soma contribute to the maintenance of a genetic lineage in different ways. The germ line transmits genetic information between generations, while the soma manifests the effects of natural selection to

act as a vehicle to carry, and mediate transmission of, the germ line. While both germ line and soma are derived simultaneously from a fertilized egg, the linkage between their ontogeny is entirely different in organisms with regulative PGCs or PGCs that are predetermined. In the former case the development of germ line and soma are tightly intertwined. Both are derived from a common pool of precursor cells that acquire their specific identities as development proceeds. Thus, the establishment of the germ line requires that presumptive PGCs receive an appropriate signaling input from the surrounding soma. [For example, bone morphogenetic proteins are required to produce PGCs in mice (Lawson et al., 1999; Ying and Zhao, 2001; Ying et al., 2001) and axolotls (A.D. Johnson, unpublished).] Because the absence of these signals would drive potential PGCs towards a somatic fate, the evolution of the somatic tissues that produce these signals is constrained. From this perspective, a major consequence of the evolution of germ plasm is to uncouple the development of the germ line from the soma, i.e. acquire weak linkage, and thereby relieve constraints.

In embryos with predetermined germ cells the precursors of PGCs are distinct from those which give rise to the soma almost from the inception of development because only they contain germ plasm. Also, germ plasm inhibits signaling inputs to the PGCs from the surrounding somatic cells, further diminishing the interaction between germ line and soma. We view this uncoupling of germ line and soma as the acquisition of a more robust somatic development in that mutations that affect somatic development could be accumulated without an effect on the germ line. As an example of this we have previously shown how the acquisition of predetermined germ cells could lead to a complete reorganization of the primitive vertebrate *bauplan*, as observed in the relatively anteriorized body plans of anuran amphibians and some teleost fish (Johnson et al., 2003b), a process we termed morphological release. Consistent with this, it has been shown that the anterior expression limits of many zebrafish hox genes have been moved toward the anterior of the body axis (van der Hoeven et al., 1996; Prince et al., 1998; Morin-Kensicki et al., 2002). This anteriorized body plan is incompatible with regulative PGC specification, which normally occurs in the posterior region of vertebrate embryos. In light of this, the observations that we report here, concerning the greater evolvability of species containing germ plasm, are not surprising. Rather, they would be predicted on the grounds of conventional interpretations of the role of development in evolution, which allow the soma to evolve more rapidly due to weak linkage with germ line development. Moreover, Kitano (2004) has recently argued that "evolution often selects traits that might enhance the robustness of the organisms that have evolved." In the current context this would suggest that selective pressures would favor the independent evolution

of germ plasm in diverse animal lineages, which we have shown here.

6. Testable sister-groups

Additional tests of the clade asymmetry hypothesis of PGC determination are difficult to find. It requires sister-groups, each possessing a different mode. We note some possibilities below and encourage collection of these missing data. Diptera (flies, 151,000 species) is the sister to a Mecoptera (scorpionflies, 550)–Siphonaptera (fleas, 2400) clade (number of species from Brusca and Brusca, 2003; sister clade data from Kristensen, 1991). An alternative phylogenetic arrangement from Gullan and Cranston (1994) suggested Diptera–Siphonaptera as sister taxa. Studied dipterans exhibit the predetermined mode and it may be that siphonapterans also exhibit the same mode (Kessel, 1939), but there is no information on the mecopterans. If the two pre-formed groups are sister taxa (sensu Gullan and Cranston, 1994), then the asymmetry would be approximately 1,53,400 to 500, if mecopterans are shown to possess the induced mode. The Nematoda (round worms, 25,000) is the sister to the Nematomorpha (hair worms, 320) (Brusca and Brusca, 2003). Studied nematodes exhibit the predetermined mode but again the sister clade is unstudied; here too asymmetry in clade size is apparent. There are other groups that may yield sister-group comparisons to test the PGC asymmetry hypothesis. Possibilities include within sharks and rays, within flatworms, within arthropods, and within lizards and snakes (Hubert, 1985).

PGC determination mechanism, based on the data and criteria we employed, represents examples of developmental constraint and constraint release. A significant challenge confronted by all evolution and development studies is the conclusion of generality extrapolated from exemplar species (Hanken, 1993). We hope that others will test the generality of our method and the constraint/release hypothesis about PGC determination mechanisms and in doing so escape the uncertainty associated with an exemplar approach. Our method requires multiple well-corroborated phylogenies and provides the escape. We do not suggest that all sister clade asymmetry is due to germ cell determination. Nonetheless, we think that germ cells are critical enough to a lineage's survival that the timing of determination and/or their position in the developing embryo is a good candidate for a constraint or a release on morphological innovation in vertebrates, and possibly across metazoans.

Acknowledgments

References

Alberch, P., 1980. Ontogenesis and morphological diversification. Amer. Zool. 20, 653–667.

Arnold, S.J., 1989. Group report: how do complex organisms evolve? In: Wake, D.B., Roth, G. (Eds.), Complex Organismal Functions: Integrations and Evolution in Vertebrates. Wiley, Chichester, pp. 403–433.

Arthur, W., 1997. The Origin of Animal Body Plans. Cambridge University Press, Cambridge.

Bachvarova, R.F., Masi, T., Drum, M., Parker, N., Mason, K., Patient, R., Johnson, A.D., 2004. Gene expression in the axolotl germ line: *Axdazl*, *Axvh*, *Axoct-4*, and *Axkit*. Dev. Dyn. 231, 871–880.

Barraclough, T.G., Nee, S., Harvey, P.H., 1998. Sister-group analysis in identifying correlates of diversification. Evol. Ecol. 12, 751–754.

Blackstone, N.W., Jasker, B.D., 2003. Phylogenetic considerations of clonality, coloniality, and mode of germline development in animals. J. Exp. Zool. (Mol. Dev. Evol.) 297B, 35–47.

Blackwell, T.K., 2004. Germ cells: finding programs of mass repression. Curr. Biol. 14, R229–R230.

Bodmer, R., 1993. The gene *tinman* is required for specification of the heart and visceral muscles in *Drosphilia*. Development 118, 719–729.

Brooks, D.R., McLennan, D.A., 1992. Phylogeny, Ecology, and Behavior: A Research Program in Comparative Biology. University Chicago Press, Chicago.

Brooks, D.R., McLennan, D.A., 2002. The Nature of Diversity: An Evolutionary Voyage of Discovery. University Chicago Press, Chicago.

Brusca, R.C., Brusca, G.J., 2003. Invertebrates. Sinauer Associates, Inc., Sunderland.

Buss, L.W., 1983. Evolution, development, and the units of selection. Proc. Natl Acad. Sci. USA 80, 1387–1391.

Buss, L.W., 1988. Diversification and germ line determination. Paleobiology 14, 313–321.

Chow, R.L., Altmann, C.R., Lang, R.A., Hemmati-Brivanlou, A., 1999. *Pax6* induces ectopic eyes in a vertebrate. Development 126, 4213–4222.

Dawkins, R., 1989. The evolution of evolvability. In: Langton, C.G. (Ed.), Artificial Life. Addison-Wesley, Reading, MA, pp. 201–220.

Erwin, D.H., Karakauer, D.C., 2004. Insights into innovation. Science 304, 1117–1119.

Extavour, C.G., Akam, M., 2003. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development 130, 5869–5884.

Farris, J.S., 1976. Expected asymmetry of phylogenetic trees. Syst. Zool. 25, 196–198.

Gullan, P.J., Cranston, P.S., 1994. The Insects. An outline of Entomology. Chapman & Hall, London.

Guyer, C., Slowinski, J.B., 1993. Comparisons of observed phylogenetic topologies with null expectations among three monophyletic lineages. Evolution 45, 340–350.

Halanych, K.M., 2004. The new view of animal phylogeny. Annu. Rev. Ecol. Evol. Syst. 35, 229–256.

Halder, G., Callaerts, P., Gehring, W.J., 1995. Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. Science 267, 1788–1792.

Hanken, J., 1993. Model systems versus outgroups: alternative approaches to the study of head development and evolution. Am. Zool. 33, 448–456.

Hashimoto, Y., Maegawa, S., Nagai, T., Yamaha, E., Suzuki, H., Yasuda, K., Inoue, K., 2004. Localized maternal factors are required for zebrafish germ cell formation. Dev. Biol. 268, 152–161.

Harvey, P.H., Pagel, M., 1991. The Comparative Method in Evolutionary Biology. Oxford University Press, Oxford.

Hennig, W., 1966. Phylogenetic Systematics. University of Illinois Press, Urbana, Il.

Houston, D.W., King, M.L., 2000. Germ plasm and molecular determinants of germ cell fate. Curr. Top. Dev. Biol. 50, 155–181.

Hubert, J., 1985. The origin and development of oocytes. In: Gans, C., Billet, F., Maderson, P.F.A. (Eds.), Biology of the Reptilia. Development A, 14. Wiley, Chichester, pp. 41–74.

Johnson, A.D., Bachvarova, R.F., Drum, M., Masi, T., 2001. Expression of axolotl *DAZL* RNA, a marker of germ plasm: widespread maternal RNA and onset of expression in germ cells approaching the gonad. Dev. Biol. 234, 402–415.

Johnson, A.D., Bachvarova, R.F., Crother, B.I., White, M.E., Patient, R., Drum, M., Masi, T., 2003a. Regulative germ cell specification in axolotl embryos: a primitive trait conserved in the mammalian lineage. Phil. Trans. R. Soc. London 358, 1371–1379.

Johnson, A.D., Drum, M., Bachvarova, R.F., Masi, T., White, M.E., Crother, B.I., 2003b. Evolution of predetermined germ cells in vertebrate embryos: implications for macroevolution. Evol. Dev. 5, 414–431.

Kessel, E.L., 1939. The embryology of fleas. Smithson.Misc. Collect. 98, 1–78.

Kirschner, M., Gerhart, J., 1998. Evolvability. Proc. Natl Acad. Sci. USA 95, 8420–8427.

Kitano, H., 2004. Biological robustness. Nat. Rev. Genet. 5, 826–837.

Kristensen, N.P., 1991. Phylogeny of extant hexapods. In: CSIRO (Ed.), The Insects of Australia. A Textbook for Students and Research Workers. Melbourne University Press, and Cornell University Press, Ithaca, NY, pp. 125–140.

Lawson, K.A., Hage, W.J., 1994. Clonal analysis of the origin of primordial germ cells in the mouse. Ciba Found. Symp. 182, 68–84.

Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C.V., Korving, J.P., Hogan, B.L., 1999. *Bmp4* is required for the generation of primordial germ cells in the mouse embryo. Genes Dev. 13, 424–436.

Leatherman, J.L., Jongens, T.A., 2003. Transcriptional silencing and translational control: key features of early germline development. Bioessays 25, 326–335.

Maddison, D.R., Maddison, W.P., 2000. MacClade 4: Analysis of Phylogeny and Character Evolution. Version 4.0. Sinauer Associates, Sunderland.

Maddison, W.P., Slatkin, M., 1991. Null models for the number of evolutionary steps in characters on a phylogenetic tree. Evolution 45, 1184–1197.

Manak, J.R., Scott, M.P., 1994. A class act: conservation of homeodomain protein functions. Development (Suppl), 61–71.

Maufroid, J.P., Capuron, A.P., 1985. A demonstration of cellular interactions during the formation of mesoderm and primordial germ cells in *Pleurodeles waltlii*. Differentiation 29, 20–24.

Maynard Smith, J., Burian, R., Kauffman, S., Alberch, P., Campbell, J., Goodwin, B., Lande, R., Raup, D., Wolpert, L., 1985. Developmental constraints and evolution: a perspective from the Mountain Lake conference on development and evolution. Q. Rev. Biol. 60, 265–287.

Meyer, A., Zardoya, R., 2003. Recent advances in the (molecular) phylogeny of vertebrates. Annu. Rev. Ecol. Evol. Syst. 34, 311–338.

Michael, P., 1984. Are the primordial germ cells (PGCs) in Urodela formed by the inductive action of the vegetative yolk mass? Dev. Biol. 103, 109–116.

Mooers, A., Heard, S.B., 1997. Inferring evolutionary process from phylogenetic tree shape. Q. Rev. Biol. 72, 31–54.

Morin-Kensicki, E.M., Melancon, E., Eisen, J.S., 2002. Segmental relationships between somites and vertebral column in zebrafish. Development 129, 3851–3860.

Nelson, J.S., 1994. Fishes of the World. Wiley, New York.

Nieuwkoop, P.D., 1969. The formation of the mesoderm in urodelean amphibians. I. Induction by the endoderm. Wilhelm Roux' Arch. 162, 341–373.

Onuma, Y., Takahashi, S., Asashima, M., Kurata, S., Gehring, W.J., 2002. Conservation of *Pax6* function and upstream activation by Notch signaling in eye development of frogs and flies. Proc. Natl Acad. Sci. USA 99, 2020–2025.

Pough, F.H., Andrews, R.M., Cadle, J.E., Crump, M.L., Savitsky, A.H., Wells, K.D., 2004. Herpetology. Pearson Prentice Hall, Upper Saddle River.

Prince, V.E., Joly, L., Ekker, M., Ho, R.K., 1998. Zebrafish hox genes: genomic organization and modified colinear expression patterns in the trunk. Development 125, 407–420.

Raff, R.A., 1996. The Shape of Life: Genes, Development and the Evolution of Animal Form. University of Chicago Press, Chicago.

Ransick, A., Cameron, R.A., Davidson, E.H., 1996. Postembryonic segregation of the germ line in sea urchins in relation to indirect development. Proc. Natl Acad. Sci. 93, 6759–6763.

Richardson, M.K., Jeffery, J.E., Coates, M.I., Bininda-Emonds, O.R.P., 2001. Comparative methods in developmental biology. Zoology 104, 278–283.

Schlichting, C.D., Murren, C.J., 2004. Evolvability and the raw materials for adaptation. In: Cronk, Q.C.B., Whitton, J., Ree, R.H., Taylor, I.E.P. (Eds.), Plant Adaptation: Molecular genetics and ecology. NRC research Press, Ottawa, pp. 18–29.

Schwenk, K., Wagner, G.P., 2003. Constraint. In: Hall, B.K., Olson, W.M. (Eds.), Keywords and concepts in evolutionary developmental biology. Harvard University Press, Cambridge, pp. 52–61.

Schwenk, K., Wagner, G.P., 2004. The relativism of constraints on phenotypic evolution. In: Pigliucci, M., Preston, K. (Eds.), Phenotypic Integration. Studying the Ecology and Evolution of Complex Phenotypes. Oxford University Press, Cambridge, pp. 390–408.

Slowinski, J.B., Guyer, C., 1989. Testing the stochasticity of patterns of organismal diversity: an improved null model. Am. Nat. 134, 907–921.

Sommer, R.J., 1999. Convergence and the interplay of evolution and development. Evol. Dev. 1, 8–10.

Sutasurya, L.A., Nieuwkoop, P.D., 1974. The induction of the primordial germ cells in the urodeles. Wilhelm Roux' Arch. 175, 199–220.

Tam, P.P., Zhou, S.X., 1996. The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. Dev. Biol. 178, 124–132.

Vamosi, S.M., Vamosi, J.C., 2005. Endless tests: guidelines for analyzing non-nested sister-group comparisons. Evol. Eco. Res. 7, 567–579.

van der Hoeven, F., Sordino, P., Fraudeau, N., Izpisua-Belmonte, J.-C., Duboule, D., 1996. Teleost *HoxD* and *HoxA* genes: comparisons with tetrapods and functional evolution of the *HoxD* complex. Mech. Dev. 54, 9–21.

Wagner, A., 2005. Robustness, evolvability, and neutrality. FEBS Lett. 579, 1772–1778.

Wagner, G.P., Müller, G.B., 2002. Evolutionary innovations overcome ancestral constraints: a re-examination of character evolution in male sepsid flies (Diptera: Sepsidae). Evol. Dev. 4, 1–6.

Wagner, G.P., Schwenk, K., 2000. Evolutionarily stable configurations: functional integration and the evolution of phenotypic stability. Evol. Biol. 31, 155–217.

Wilkins, A.S., 2002. The Evolution Of Developmental Pathways. Sinauer Associates, Sunderland.

Ying, Y., Zhao, G.Q., 2001. Cooperation of endoderm-derived *BMP2* and extraembryonic ectoderm-derived *BMP4* in primordial germ cell generation in the mouse. Dev. Bio. 232, 484–492.

Ying, Y., Qi, X., Zhao, G.Q., 2001. Induction of primordial germ cells from murine epiblasts by synergistic action of *BMP4* and *BMP8B* signaling pathways. Proc. Natl Acad. Sci. 98, 7858–7862.