JOURNAL OF EXPERIMENTAL ZOOLOGY (MOL DEV EVOL) 304B:150–158 (2005)

In Search of the Vertebrate Phylotypic Stage: A Molecular Examination of the Developmental Hourglass Model and von Baer's Third Law

EINAT HAZKANI-COVO[1], DAVID WOOL[1], AND DAN GRAUR[1,2,*]
[1]*Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel*
[2]*Department of Biology and Biochemistry, University of Houston, Houston, Texas 77204*

ABSTRACT In 1828, Karl von Baer proposed a set of four evolutionary ''laws'' pertaining to embryological development. According to von Baer's third law, young embryos from different species are relatively undifferentiated and resemble one another but as development proceeds, distinguishing features of the species begin to appear and embryos of different species progressively diverge from one another. An expansion of this law, called ''the hourglass model,'' has been proposed independently by Denis Duboule and Rudolf Raff in the 1990s. According to the hourglass model, ontogeny is characterized by a starting point at which different taxa differ markedly from one another, followed by a stage of reduced intertaxonomic variability (the phylotypic stage), and ending in a von-Baer-like progressive divergence among the taxa. A possible ''translation'' of the hourglass model into molecular terminology would suggest that orthologs expressed in stages described by the tapered part of the hourglass should resemble one another more than orthologs expressed in the expansive parts that precede or succeed the phylotypic stage. We tested this hypothesis using 1,585 mouse genes expressed during 26 embryonic stages, and their human orthologs. Evolutionary divergence was estimated at different embryonic stages by calculating pairwise distances between corresponding orthologous proteins from mouse and human. Two independent datasets were used. One dataset contained genes that are expressed solely in a single developmental stage; the second was made of genes expressed at different developmental stages. In the second dataset the genes were classified according to their earliest stage of expression. We fitted second order polynomials to the two datasets. The two polynomials displayed minima as expected from the hourglass model. The molecular results suggest, albeit weakly, that a phylotypic stage (or period) indeed exists. Its temporal location, sometimes between the first-somites stage and the formation of the posterior neuropore, was in approximate agreement with the morphologically defined phylotypic stage. The molecular evidence for the later parts of the hourglass model, i.e., for von Baer's third law, was stronger than that for the earlier parts. *J. Exp. Zool. (Mol. Dev. Evol.) 304B:150–158, 2005.* © 2005 Wiley-Liss, Inc.

INTRODUCTION

Attempts to uncover rules linking embryonic development with evolutionary processes can be traced back to Karl von Baer, who in 1828 proposed four laws of animal development. These laws assert that (1) general features of the embryo appear earlier than special features, (2) special characters develop from general characters, (3) embryos of different species progressively diverge from one another during ontogeny, and (4) embryos of one animal can never resemble the adult form of another animal, but only its embryo (von Baer, 1828; Gould, '77). For purposes of clarity, in this note we shall primarily deal with the third law. Interestingly, von Baer discovered his revolutionary laws because of a laboratory mishap. ''In my possession are two little embryos in spirit, whose names I have omitted to attach, and at present I am quite unable to say to what class they belong. They may be lizards, or small birds, or very young mammalia, so complete is the

*Correspondence to: Dan Graur, Department of Biology and Biochemistry, University of Houston, Houston, Texas 77204. E-mail: dgraur@uh.edu
Received 21 October 2004; Accepted 10 December 2004
Published online 7 March 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21033

© 2005 WILEY-LISS, INC.

similarity in the mode of formation of the head and trunk of these animals.''

von Baer's third law remained unchallenged (albeit frequently misattributed to Ernst Haeckel) until Seidel ('60), Sander ('83), and Elinson ('87) noticed that the law does not apply to the earliest stages of development. In vertebrates, for instance, the gastrulation stages can differ widely even among closely related species. The progressive divergence model was, therefore, replaced by the hourglass (Raff, '96) or egg-timer model (Duboule, '94), in which ontogeny is characterized by a starting point in which different taxa differ markedly from one another, followed by a stage of reduced intertaxonomic variability, and finally by a progressive divergence among the taxa. Thus, the developmental hourglass model predicts a midriff constriction, i.e., a developmental stage in which a maximum degree of similarity among the members of the phylum is reached (Hall, '97). This stage has been variably called ''körpergrundgestalt'' (Seidel, '60), ''phylotypic stage'' (Sander, '83), or simply ''phylotype'' (Slack et al., '93). (Since the term ''phylotype'' is also used in bacterial systematics as a synonym for the term ''species'' when morphological and physiological descriptions are missing, we shall use from now the term ''phylotypic stage.'') According to the developmental hourglass, the similarity among organisms belonging to a higher taxon is higher at the phylotypic stage than at either earlier or later stages of ontogenical development (Fig. 1).

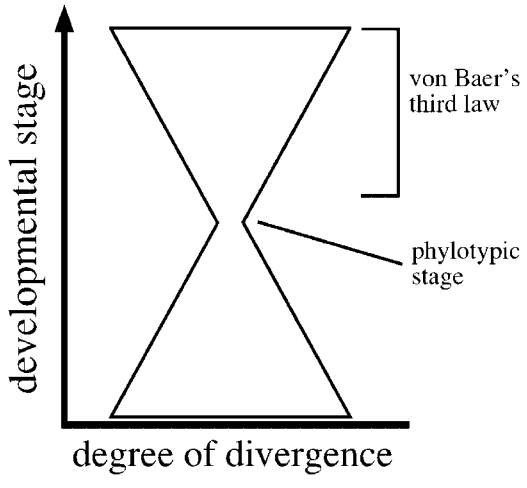

Fig. 1. Schematic representation of the hourglass model for the relationship between evolution and development. Horizontal distance represents morphological or molecular divergence during evolution and vertical distance represents developmental stage. The bottleneck of the hourglass represents the phylotypic stage. None of the axes are scaled.

The exact temporal positioning of the phylotypic stage within embryonic progression is far from consensual. The vertebrate phylotypic stage is currently thought to occur sometimes after the completion of the major morphogenetic movements during gastrulation, between the headfold stage and the tailbud stage (Duboule, '94). In the literature, three developmental stages are mostly identified with the phylotypic stage. Ballard ('81) identified the pharyngula as the vertebrate phylotypic stage. The pharyngula is the earliest stage in which four distinguishing features (i.e., notochord, dorsal hollow nerve cord, post-anal tail, and a series of paired branchial grooves or gill slits) are present. Wolpert ('91) defined the early stage of somite segregation just after neurulation as the phylotypic stage, while Slack et al. ('93) suggested the tailbud stage. The tailbud stage is also suggested from Haeckel's famous (or infamous) drawings (Haeckel, 1874). Richardson ('95) suggested using the term ''phylotypic period'' instead of ''phylotypic stage,'' thus, avoiding the connotation of a single time point implied by the word ''stage.''

The existence of a phylotypic stage in vertebrates is broadly accepted in developmental biology (reviewed in Richardson and Keuck, 2002). However, this concept has also been questioned (Richardson et al., '97; Collazo, 2000; Bininda-Emonds et al., 2003). The criticism was based on detailed morphological data that seem to suggest that a large degree of variation exists in the stages commonly suspected to make up the phylotypic stage. Poe and Wake (2004) have recently suggested that the hourglass model is ''unnecessarily complex'' and, therefore, a simpler model that postulates that evolutionary changes are easier between ontogenetically adjacent events is adequate to explain the morphological divergence during evolution. For a discussion of the pro- and anti-phylotypic attitudes, see Sander and Schmidt (2004).

Raff ('92, '96) has suggested a mechanistic rationale for the existence of a phylotypic stage. According to this explanation, the web of intense interactions among organ primordia (somites, neural tube and chorda) that exist in the phylotypic stage causes any small mutational change to result in deleterious pleiotropic effects in the embryo. At earlier stages there are fewer interactions as there are no organ primordia yet. At later stages there are many more interactions, but they take place within semi-independent modules (e.g., limbs, lungs), which limit the effects of mutational

changes to only parts of the organism. Indeed, Galis and Metz (2001) and Galis et al. (2002) claimed that embryos at the phylotypic stage are more vulnerable to mutation than any other stages. By analyzing teratological and mutational data from vertebrates, they showed that changes during the phylotypic stage have pleiotropic effects that lead to multiple abnormalities.

Originally a purely morphological concept, the idea of a phylotypic stage has been expanded to include molecular data. The involvement of genes, such as the *Hox* genes, in axis formation lead to the suggestion that genes and expression patterns are responsible for the hourglass pattern of divergence (Slack et al., '93; Duboule, '94; Yost, '99). Slack et al. ('93) coined the term zootype to denote a particular spatial pattern of gene expression in the organism. The zootype of a higher taxon is most clearly identifiable at the phylotypic stage.

In the literature, von Baer's third law and the hourglass model have been examined extensively at the morphological level. So far, however, very little attention was paid to the verification of these models at the molecular level. The only exception we are aware of appears in a nearly forgotten paper by Ivanov ('87), in which parts of von Baer's laws and Haeckel's law of recapitulation are tested on globin sequence data from mammals.

Our very simple molecular rendition of the hourglass model hypothesizes that orthologs expressed in stages described by the tapered part of the hourglass should resemble one another more closely than orthologs expressed in the expansive parts that precede and succeed the phylotypic stage (Fig. 1). Thus, evolutionary distances should decrease from the zygote to the phylotypic stage, at which point the distances should reach a minimum. After the phylotypic stage, we expect the genetic distances to increase monotonically for the rest of the embryonic development. Thus, the aims of this note are to confirm that a phylotypic stage indeed exists and, if possible to position it, at least approximately, on a temporal developmental sequence.

In practical terms, we examine the developmental hourglass model by using the relationship between the degree of divergence between orthologous proteins from human and mouse, on the one hand, and developmental stage in which the protein is expressed, on the other. We note, however, that by looking exclusively at orthologous gene pairs, we may be ignoring important influences exerted by factors such as paralogous subfunctionalization (Force et al., '99; Lynch and Force, 2000; Ward and Durrett, 2004), DNA factors (Arnone and Davidson, '97; Wray, 2003; Wray et al., 2003; Bejerano et al., 2004; Wagner et al., 2004; Woolfe et al., 2004), or protein interaction networks (Hirsh and Fraser, 2001; Fraser et al., 2003; Krylov et al., 2003). We consider our hypothesis as the most parsimonious molecular scenario that might explain the developmental hourglass, and as such a useful approach.

DATA AND METHODS

Mouse genes expressed during various developmental stages

Data on genes expressed during particular stages of embryonic development were taken from the March 2004 version of the Gene Expression Database (GXD, www.informatics.jax.org). Only expression data from wild-type strains were used. We included expression data from 26 embryonic stages starting with the one-cell egg and ending before birth (Table 1). The raw compilation included a total of 2,860 genes and 19,216 entries containing empirical information pertaining to the developmental stages at which a gene is expressed. The developmental stages that are used by GXD were taken from the Edinburgh Mouse Atlas Project (EMAP, Baldock et al., 2003), which is based on Theiler ('89). We selected all genes that were reported to have positive expression in one or more stages. In a particular embryonic stage, a gene may be expressed in some anatomical structure but not in another. For our needs, a gene is considered as ''expressed'' if expression was observed at least once, regardless of tissue or method of detection. The number of genes fulfilling this condition was 2,656.

Human orthologs, protein identities, and protein distances

Decisions concerning orthology between mouse and human genes were adopted from GXD (ftp://ftp.informatics.jax.org/pub/reports/HMD_HumanSequence.rpt). Sequences of the mouse proteins encoded by the genes in GXD and their human orthologs were taken from the February 2004 version of SwissProt (ftp://ftp.expasy.org/databases/uniprot/knowledgebase/uniprot_sprot.fasta) and the January 2004 version of RefSeq (ftp://ftp.ncbi.nih.gov/refseq/H_sapiens/mRNA_Prot/hs.faa). Since we only used genes with known human orthologs whose accession numbers were found in

TABLE 1. Prenatal developmental stages derived from the Edinburgh Mouse Atlas Project. Stages marked in gray were tested for possible equivalence with the phylotypic stage. Data on stage 27 is not available in GXD. dpc, days post conception

Stage	dpc	Developmental status
1	0–0.9	One cell egg
2	1	Beginning of cell division
3	2	Morula
4	3	Advanced division and segmentation
5	4	Blastocyst
6	4.5	Implantation
7	5	Formation of egg cylinder
8	6	Differentiation of egg cylinder
9	6.5	Advanced endometrial reaction, gastrulation starts
10	7	Amnion
11	7.5	Neural plate, early headfold, presomite stage
12	8	First somites; late headfold
13	8.5	Turning of the embryo
14	9	Formation and closure of anterior neuropore
15	9.5	Formation of posterior neuropore, forelimb bud
16	10	Closure of posterior neuropore, hindlimb and tailbud
17	10.5	Deep lens indentation
18	11	Closure of lens vesicle
19	11.5	Complete separation of lens vesicle
20	12	Earliest sign of fingers
21	13	Anterior footplate indented
22	14	Fingers separate distally
23	15	Toes separate
24	16	Reposition of umbilical hernia
25	17	Joining of fingers and toes
26	18	Long whiskers
27	19	Newborn mouse

either SwissProt or RefSeq, our orthologous dataset consisted of 1,599 pairs of proteins.

The percentage of amino acid identity between each pair was calculated using the BLAST 2 Sequences program with the BLOSUM80 substitution matrix and without the default option of low complexity masking (Tatusova and Madden, '99).

Alignment for each pair of mouse-human orthologs was performed using ClustalW (Higgins et al., '96). Protein distances were calculated with the PROTDIST program from the PHYLIP package (Felsenstein, '93) using the default JTT matrix. In our analysis we ignored 14 protein pairs whose protein distance is higher than 1 (100 PAM or 57% observed distance). Our final dataset included 1,585 pairs of proteins.

Testing the hourglass model

Genes may be expressed during several developmental stages. Thus, gene expression data may contain dependent variables. To steer clear of this hurdle, we use two datasets consisting of independent entries. Dataset I contains 356 genes that are expressed solely in a single developmental stage. Dataset II contains 1,585 genes. In this dataset, each gene was assigned to the first developmental stage in which it is expressed. Two analyses were performed for each dataset (see below).

(a) Fitting a parabola model to data

A second degree polynomial model of the form $y=ax+b$ was fitted to the data by using the least squares method (Harter, '83). The independent variable x is the number of days post conception (*dpc*). The *dpc* values were from Thieler ('89). When a range rather than a single value was reported for a stage, the mid *dpc* value was used. The dependent variable y is the corresponding mouse-human distance between the relevant orthologs. If the hourglass model is correct, we should obtain a parabola with the phylotypic stage at the minimum.

Residual distribution and r^2 statistics were used to evaluate the fit of the model to the data. The residuals are defined as the difference of the predicted values of y according to the model (the predicted distance that is calculated at each developmental stage) and the observed values of y (the actual distances that are found at each developmental stage). Randomly distributed residuals would support the model. r^2 measures the success of the fit in explaining the observed variation in the data. If the hourglass model is correct, we expect the second order polynomials to fit the data well.

(b) Analysis of suspected phylotypic stages

Based on the literature and allowing for large margins of error, we decided to test eight developmental stages for possible equivalence with the phylotypic stage. For simplicity, in the following we shall refer to these stages as ''suspects'' (Table 1). The analyses were carried out separately for each suspect in each of the two datasets, for a total of 16 analyses (Table 2). In each analysis, we divided the data into two subsets. The first subset included all stages from stage 1 to (and including) the suspect stage. The second subset included all remaining stages to stage 26. For each subset, we performed a Spearman nonparametric correlation test (Sokal and Rohlf, '95) between the protein distances and the stage number. If the hourglass model is correct, we expect that the correlations

TABLE 2. Spearman correlation tests for different developmental stages

Suspect stage	*dpc*	Dataset I		Dataset II	
		Up to and including suspect stage (left-handed subset)	After suspect stage (right-handed subset)	Up to and including suspect stage (left-handed subset)	After suspect stage (right-handed subset)
10	7	n = 11	n = 345	n = 353	n = 1232
		r = 0.428	r = 0.014	r = −0.046	r = 0.064
		p = 0.189	p = 0.791	p = 0.384	p = 0.024
11	7.5	n = 40	n = 316	n = 461	n = 1124
		r = −0.111	r = 0.025	r = −0.024	r = 0.077
		p = 0.494	p = 0.657	p = 0.604	p = 0.010
12	8	n = 43	n = 313	n = 523	n = 1062
		r = 0.102	r = 0.050	r = −0.061	r = 0.060
		p = 0.515	p = 0.376	p = 0.165	p = 0.051
13	8.5	n = 46	n = 310	n = 648	n = 937
		r = 0.004	r = 0.042	r = −0.032	r = 0.069
		p = 0.979	p = 0.459	p = 0.410	p = 0.034
14	9	n = 49	n = 307	n = 678	n = 907
		r = 0.058	r = 0.053	r = −0.040	r = 0.062
		p = 0.694	p = 0.354	p = 0.300	p = 0.060
15	9.5	n = 52	n = 304	n = 803	n = 782
		r = 0.067	r = 0.061	r = −0.014	r = 0.082
		p = 0.636	p = 0.293	p = 0.698	p = 0.021
16	10	n = 54	n = 302	n = 856	n = 729
		r = 0.099	r = 0.070	r = 0.011	r = 0.118
		p = 0.475	p = 0.224	p = 0.752	p = 0.001
17	10.5	n = 64	n = 292	n = 935	n = 650
		r = −0.036	r = 0.059	r = −0.017	r = 0.098
		p = 0.775	p = 0.316	p = 0.606	p = 0.013

for the subsets to the left and the right of the phylotypic stage to yield negative and positive coefficients, respectively. We may, then, identify the phylotypic stage on the basis of the absolute values and the statistical significance of the correlation coefficients for the eight suspects.

Control dataset

We used Homologene (ftp://ftp.ncbi.nih.gov/pub/HomoloGene/build37.2/homologene.xml.gz, Wheeler et al., 2004) as a source for mouse-human protein distances. The database includes 15,917 mouse-human protein pairs and their distances. Genes included in this list represent all mouse and human genes, rather then only developmental genes. The pairs are determined either by extraneous information on orthology or according to reciprocal best Blast matches. All distances derived from this dataset were divided randomly among the 26 developmental stages according to the proportions of genes in each stage in dataset II. The two analyses for testing the hourglass model were, then, applied to this control dataset.

RESULTS

Our mouse-human orthologous dataset included 1,585 proteins. The mean amino-acid sequence identity for this dataset was $87 \pm 11\%$ and the median identity was 90%. The mean and median protein distances were 0.16 ± 0.16 and 0.10, respectively.

Second-degree polynomials were fitted to the two datasets. With x measured in number of days post conception and y in orthologous protein distances, the equations that were obtained for datasets I and II were $y = 0.0001x^2 - 0.0022x + 0.1778$ and $y = 0.0005x^2 - 0.0079 + 0.1817$, respectively.

As expected from the hourglass model, both fitted parabolas had minima. The minimum of the parabola described in the equation for dataset I appears at 9.64 *dpc*, or approximately at developmental stage 15 (Table 1). The minimum of the

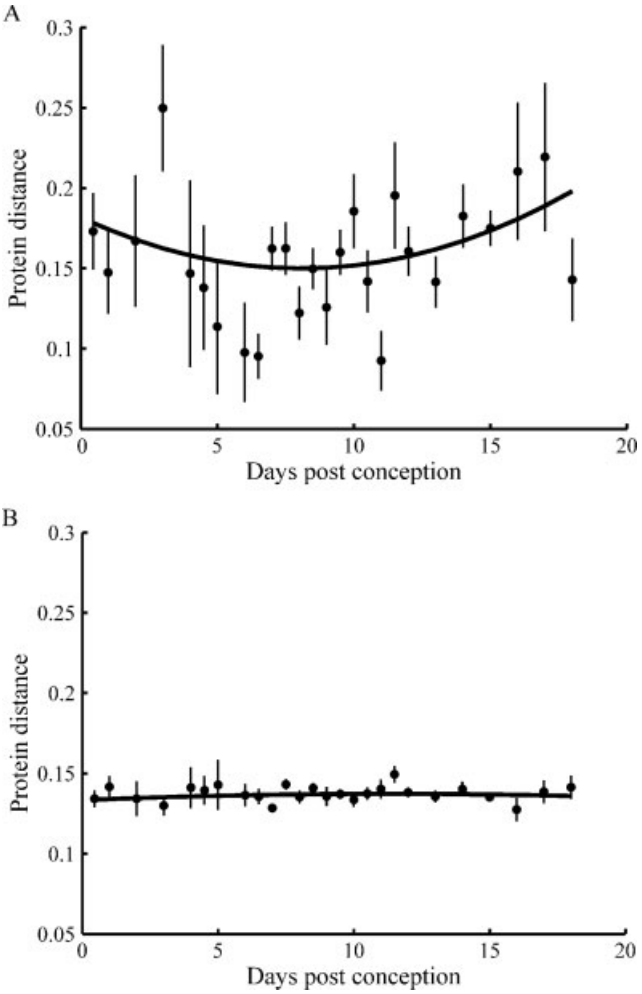

Fig. 2. Relationship between protein distances and developmental stage expressed in days post conception. (A) Mean protein distances ± standard errors of 1,585 protein-pair distances from dataset II at 26 developmental stages are shown as vertical bars. The best fitted second-order polynomial exhibiting a putative hourglass behavior is shown. (B) Mean protein distances ± standard errors of 15,917 protein-pair distances from the random dataset (see Data and Methods). The best-fitted second order polynomial does not exhibit hourglass behavior.

parabola fitted to dataset II appears at 8.07 *dpc*, or approximately at developmental stage 12. The parabola fitted to the largest dataset (dataset II) is shown in Figure 2A. Residual distribution and r^2 statistic were used to evaluate the fit of the parabolas to the data. A nonrandom residual distribution was found, i.e., the model fits the data poorly. Results obtain from the random dataset are shown in Figure 2B. The random dataset did not exhibit a minimum.

An additional and more flexible analysis was performed to identify the phylotypic stage. In this analysis it was not necessary to force a fit of all orthologous distances to a single model in order to define the hourglass. Instead, the analysis focused only on the phylotypic period, trying to find the best two-fold subdivision of the developmental stages. For each of the eight suspect stages listed in Table 1, two subsets were analyzed by Spearman correlations (see Data and Methods). The first subset included all stages from stage 1 to (and including) the suspect. The second subset included all remaining stages to stage 26. We expect the correlations for the first subsets to yield negative coefficients and the correlations for the second subsets to yield positive coefficients.

Spearman nonparametric correlation coefficients between protein distance and developmental stage are shown in Table 2. We note that the results obtained from dataset I are not statistically significant. Most of the correlation coefficients obtained were positive, except for two subsets on the left of the suspected putative phylotypic stage. The left-hand subsets of dataset II yielded no statistical significant correlation coefficients. Most subsets on the right of the suspected phylotypic stage, however, yielded statistically significant coefficients. As expected, the correlation coefficients in the subsets to the left of the putative phylotypic stage are mostly negative, whereas those to the right of the suspects yielded only positive correlation coefficients. We could not find any ''suspect'' showing a statistically significant negative correlation for the left-hand subset and a statistically significant positive correlation for the right-hand subset. The differences in correlation coefficients between the right-hand subsets and the left-hand subsets ranged from 0.10 at stage 15 to 0.12 at stage 12. In all cases, only a very small fraction of the variability in degrees of evolutionary conservation is explainable by developmental stage. No statistically significant correlation coefficients were found in the analyses involving the random dataset. The signs of the correlation coefficients were opposite to those for dataset II, i.e., mostly positive correlations were found in the left-handed subset and mostly negative correlations were found in the right-handed subset.

DISCUSSION

The present study represents, to the best of our knowledge, the first attempt to test the hourglass model from a molecular perspective. According to the developmental hourglass, ontogeny is characterized by a starting point at which different taxa

differ markedly from one another, followed by a phylotypic stage of reduced intertaxonomic variability, and finally by a progressive divergence among the taxa (Fig. 1). Assuming a simple relationship between phenotype and genotype, we hypothesized that the molecular divergence of genes expressed in sequential stages during early embryonic life decreases gradually, reaching a minimum at the phylotypic stage. Subsequently, molecular divergence will increase. In our analyses we assumed that a gene that is expressed at a particular developmental stage in the mouse is also expressed at the same stage in human.

Both mean and median percentage of identity found here are somewhat higher than those reported by the Mouse Genome Sequencing Consortium (Waterston et al., 2002). The consortium used 12,845 mouse-human orthologous pairs for which lineage-specific duplications do not seem to have occurred in either lineage. The mean orthologous identity that was reported was 70% and the median identity was 79%. In our dataset of developmental orthologs we found a mean identity of 87% and a median identity of 90%. This large difference may result from more stringent selective constraints operating on developmental genes in comparison to other gene sets. However, this result may also be indicative of a bias in the identification of orthologs. Orthologs with a high identity level are easier to detect and, hence, may appear more frequently in curated databases, such as ours.

We fitted second-degree polynomials to the data and obtained polynomials with global minima. These finding, in themselves, indicate that the hourglass model is echoed in the molecular data. According to these polynomials, the phylotypic stage appears somewhere between stages 12 and 15 (Table 1), in agreement with ranges proposed in the literature (Wolpert, '91; Slack et al., '93; Duboule, '94). Stage 12 resembles the earlier phylotypic period and is characterized by the first appearance of somite pairs and with the formation of late headfold. Stage 15 resembles the later phylotypic period and is characterized by the formation of posterior neuropore and the appearance of forelimb bud. We note, however, that the goodness of fit of these two models to the data is quite poor.

Similar conclusions were derived from the analysis of phylotypic suspects in dataset II, which includes the first stage of expression of each gene. As expected from the hourglass model, distances from stage 1 to the phylotypic suspects exhibited negative correlations while distances from the phylotypic suspect to birth exhibited positive correlation. We could not find a ''suspect'' for which both subsets gave significant results. Only the second part of the hourglass, described by von Baer's third law, showed a statistically significant relation between evolutionary conservation and developmental stage. The difference in correlation coefficients between any two subsets suggests equal support of all ''suspects.'' These results may be interpreted as supporting a somewhat diffuse ''phylotypic period'' (Richardson, '95) rather than a single stage.

Admittedly, by using molecular data we could not find undisputable statistical evidence for the hourglass model. Only a small fraction of variation in evolutionary conservation was explainable by developmental stage. We note, however, that the evidence for the later parts of the hourglass model, i.e., for von Baer's third law, is a little stronger. If we adopt a formalistic approach, our findings may be construed as supporting some morphological studies that were unable to provide quantitative evidence for the hourglass model (Richardson et al., '97; Bininda-Emonds et al., 2003). However, the mere fact that the relationship between gene sequence conservation and developmental stage exhibits a minimum may be taken as supportive of the hourglass model, more so since in our analyses we lump together proteins of different function and different selective constraints. The pattern observed with the control dataset is completely different. Finally, we note that we have used conservative methods of nonparametric analysis, and this approach may have unduly reduced statistical power.

Our results suggest that a phylotypic stage may exist, but that developmental and evolutionary factors may have conspired to camouflage its temporal identification. First, proteins at different developmental stages might have different functions and different selective constraints. These factors might have a stronger impact on sequence conservation than the developmental stage. Therefore, a priori impediments may exist when trying to identify evolutionary patterns in proteins, whose only common denominator is developmental stage. Second, genes that are expressed during development are more conserved than the overall mouse-human orthologous repertoire. If the reason for this finding is a more stringent selection on developmental genes than on other gene sets, the entire developmental gene repertoire might be extremely conserved during evolu-

tion. This reduction in variation might cause the difference in protein distances between developmental stages to be too small for the phylotypic stage to be identified at the molecular level. Third, our analysis was based on molecular data from two closely related taxa. When reliable data becomes available for more distantly related vertebrates, a more precise molecular testing of the hourglass model may be feasible. These three reasons might explain why the light shed by molecular methodology on the hourglass model of ontogenetic development was more limited than optimistically expected.

The molecular data identifies several alternative possible phylotypic stages, and these all agree with the range of stages proposed in the morphological literature. All in all, our study indicates that the hourglass model is viable and worthy of further consideration. More precisely, we regard our results as supporting von Baer's septaquintaquinquecentennial model more strongly than the decennial hourglass. Notwithstanding, we must end this note by stating that unambiguously proving or disproving the hourglass model will require much more expression and sequence data than currently available.

ACKNOWLEDGMENT

We wish to thank Tal Dagan for her help and critical comments. E. H-C was supported in part by the Switzerland Institute for Developmental Biology.

LITERATURE CITED

Arnone MI, Davidson EH. 1997. The hardwiring of development: organization and function of genomic regulatory systems. Development 124:1851–1864.

Baldock RA, Bard JB, Burger A, Burton N, Christiansen J, Feng G, Hill B, Houghton D, Kaufman M, Rao J, Sharpe J, Ross A, Stevenson P, Venkataraman S, Waterhouse A, Yang Y, Davidson DR. 2003. EMAP and EMAGE: a framework for understanding spatially organized data. Neuroinformatics 1:309–325.

Ballard WW. 1981. Morphogenetic movements and fate maps of vertebrates. American Zoologist 21:391–399.

Bejerano G, Pheasant M, Makunin I, Stephen S, Kent WJ, Mattick JS, Haussler D. 2004. Ultraconserved Elements in the Human Genome. Science 304:1321–1325.

Bininda-Emonds OR, Jeffery JE, Richardson MK. 2003. Inverting the hourglass: quantitative evidence against the phylotypic stage in vertebrate development. Proc R Soc Lond B Biol Sci 270:341–346.

Collazo A. 2000. Developmental variation, homology, and the pharyngula stage. Syst Biol 49:3–18.

Cooke J. 2004. Developmental mechanism and evolutionary origin of vertebrate left/right asymmetries. Biol Rev Camb Philos Soc 79:377–407.

Duboule D. 1994. Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. Dev Suppl:135–142.

Elinson RP. 1987. Change in Developmental Patterns: Embryos of Amphibians with Large Eggs. In: Raff RA, Raff EC, editors. Development as an Evolutionary Process. New York: Alan R. Liss. p 1–21.

Felsenstein J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Seattle: University of Washington.

Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151:1531–1545.

Fraser HB, Wall DP, Hirsh AE. 2003. A simple dependence between protein evolution rate and the number of protein-protein interactions. BMC Evol Biol 3:11.

Galis F, Metz JA. 2001. Testing the vulnerability of the phylotypic stage: on modularity and evolutionary conservation. J Exp Zool 291:195–204.

Galis F, van Dooren TJ, Metz JA. 2002. Conservation of the segmented germband stage: robustness or pleiotropy? Trends Genet 18:504–509.

Gould SJ. 1977. Ontogeny and Phylogeny: Cambridge Mass., Belknap Press of Harvard University Press.

Haeckel E. 1874. Anthropogenie oder Entwickelungsgeschichte des Menschen. Engelmann, Leipzig.

Hall BK. 1997. Phylotypic stage or phantom: is there a highly conserved embryonic stage in vertebrates? Trends in Ecology & Evolution 12:461–463.

Harter HL. 1983. Least Squares. In: Kotz S, Johnson NL, editors. Encyclopedia of Statistical Sciences. New York: John Wiley & Sons. p 593–598.

Higgins DG, Thompson JD, Gibson TJ. 1996. Using CLUSTAL for multiple sequence alignments. Methods Enzymol 266:383–402.

Hirsh AE, Fraser HB. 2001. Protein dispensability and rate of evolution. Nature 411:1046–1049.

Ivanov OC. 1987. New evidence for validity of Haeckel's law on molecular level. Naturwissenschaften 74:40–42.

Krylov DM, Wolf YI, Rogozin IB, Koonin EV. 2003. Gene loss, protein sequence divergence, gene dispensability, expression level, and interactivity are correlated in eukaryotic evolution. Genome Res 13:2229–2235.

Lynch M, Force A. 2000. The probability of duplicate gene preservation by subfunctionalization. Genetics 154:459–473.

Poe S, Wake MH. 2004. Quantitative tests of general models for the evolution of development. Am Nat 164:415–422.

Raff RA. 1992. Evolution of developmental decisions and morphogenesis: the view from two camps. Dev Suppl:15–22.

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

Richardson MK. 1995. Heterochrony and the phylotypic period. Dev Biol 172:412–421.

Richardson MK, Hanken J, Gooneratne ML, Pieau C, Raynaud A, Selwood L, Wright GM. 1997. There is no highly conserved embryonic stage in the vertebrates: implications for current theories of evolution and development. Anat Embryol (Berl) 196:91–106.

Richardson MK, Keuck G. 2002. Haeckel's ABC of evolution and development. Biol Rev Camb Philos Soc 77:495–528.

Sander K. 1983. The evolution of patterning mechanisms: gleanings from insect embryogenesis and spermatogenesis. In: Goodwin BC, Holder N, Wylie CC, editors. Development and evolution: Cambridge University Press. p 137–159.

Sander K, Schmidt-Ott U. 2004. Evo-devo aspects of classical and molecular data in a historical perspective. J Exp Zoolog Part B Mol Dev Evol 302:69–91.

Seidel F. 1960. Körpergrundgestalt und Keimstruktur eine Erörterung über die Grundlagen der vergleichenden und experimentellen Embryologie und deren Gültigkeit bei phylogenetischen Überlegungen. Zool Anz 164:245–305.

Slack JM, Holland PW, Graham CF. 1993. The zootype and the phylotypic stage. Nature 361:490–492.

Sokal RR, Rohlf FJ. 1995. Biometry: the principles and practice of statistics in biological research. New York: W.H. Freeman and Co.

Tatusova TA, Madden TL. 1999. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol Lett 174:247–250.

Theiler K. 1989. The House Mouse: Atlas of Mouse Development. New York: Springer-Verlag.

von Baer KE. 1828. Entwicklungsgeschichte der Thiere: Beobachtung und Reflexion. Bornträger, Königsberg.

Wagner GP, Fried C, Prohaska SJ, Stadler PF. 2004. Divergence of Conserved NonCoding Sequences: Rate Estimates and Relative Rate Tests. Mol Biol Evol 21: 2116–2121.

Ward R, Durrett R. 2004. Subfunctionalization: How often does it occur? How long does it take? Theor Popul Biol 66:93–100.

Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P, Antonarakis SE, Attwood J, Baertsch R, Bailey J, Barlow K, Beck S, Berry E, Birren B, Bloom T, Bork P, Botcherby M, Bray N, Brent MR, Brown DG, Brown SD, Bult C, Burton J, Butler J, Campbell RD, Carninci P, Cawley S, Chiaromonte F, Chinwalla AT, Church DM, Clamp M, Clee C, Collins FS, Cook LL, Copley RR, Coulson A, Couronne O, Cuff J, Curwen V, Cutts T, Daly M, David R, Davies J, Delehaunty KD, Deri J, Dermitzakis ET, Dewey C, Dickens NJ, Diekhans M, Dodge S, Dubchak I, Dunn DM, Eddy SR, Elnitski L, Emes RD, Eswara P, Eyras E, Felsenfeld A, Fewell GA, Flicek P, Foley K, Frankel WN, Fulton LA, Fulton RS, Furey TS, Gage D, Gibbs RA, Glusman G, Gnerre S, Goldman N, Goodstadt L, Grafham D, Graves TA, Green ED, Gregory S, Guigo R, Guyer M, Hardison RC, Haussler D, Hayashizaki Y, Hillier LW, Hinrichs A, Hlavina W, Holzer T, Hsu F, Hua A, Hubbard T, Hunt A, Jackson I, Jaffe DB, Johnson LS, Jones M, Jones TA, Joy A, Kamal M, Karlsson EK, Karolchik D, Kasprzyk A, Kawai J, Keibler E, Kells C, Kent WJ, Kirby A, Kolbe DL, Korf I, Kucherlapati RS, Kulbokas EJ, Kulp D, Landers T, Leger JP, Leonard S, Letunic I, Levine R, Li J, Li M, Lloyd C, Lucas S, Ma B, Maglott DR, Mardis ER, Matthews L, Mauceli E, Mayer JH, McCarthy M, McCombie WR, McLaren S, McLay K, McPherson JD, Meldrim J, Meredith B, Mesirov JP, Miller W, Miner TL, Mongin E, Montgomery KT, Morgan M, Mott R, Mullikin JC, Muzny DM, Nash WE, Nelson JO, Nhan MN, Nicol R, Ning Z, Nusbaum C, O'Connor MJ, Okazaki Y, Oliver K, Overton-Larty E, Pachter L, Parra G, Pepin KH, Peterson J, Pevzner P, Plumb R, Pohl CS, Poliakov A, Ponce TC, Ponting CP, Potter S, Quail M, Reymond A, Roe BA, Roskin KM, Rubin EM, Rust AG, Santos R, Sapojnikov V, Schultz B, Schultz J, Schwartz MS, Schwartz S, Scott C, Seaman S, Searle S, Sharpe T, Sheridan A, Shownkeen R, Sims S, Singer JB, Slater G, Smit A, Smith DR, Spencer B, Stabenau A, Stange-Thomann N, Sugnet C, Suyama M, Tesler G, Thompson J, Torrents D, Trevaskis E, Tromp J, Ucla C, Ureta-Vidal A, Vinson JP, Von Niederhausern AC, Wade CM, Wall M, Weber RJ, Weiss RB, Wendl MC, West AP, Wetterstrand K, Wheeler R, Whelan S, Wierzbowski J, Willey D, Williams S, Wilson RK, Winter E, Worley KC, Wyman D, Yang S, Yang SP, Zdobnov EM, Zody MC, Lander ES. 2002. Initial sequencing and comparative analysis of the mouse genome. Nature 420:520–562.

Wheeler DL, Church DM, Edgar R, Federhen S, Helmberg W, Madden TL, Pontius JU, Schuler GD, Schriml LM, Sequeira E, Suzek TO, Tatusova TA, Wagner L. 2004. Database resources of the National Center for Biotechnology Information: update. Nucl Acids Res 32:D35–40.

Wolpert L. 1991. The Triumph of the Embryo. Oxford: Oxford University Press.

Woolfe A, Goodson M, Goode DK, Snell P, McEwen GK, Vavouri T, Smith SF, North P, Callaway H, Kelly K, Walter K, Abnizova I, Gilks W, Edwards YJ, Cooke JE, Elgar G. 2004. Highly Conserved NonCoding Sequences Are Associated with Vertebrate Development. PLoS Biol 3:e7.

Wray GA. 2003. Transcriptional regulation and the evolution of development. Int J Dev Biol 47:675–684.

Wray GA, Hahn MW, Abouheif E, Balhoff JP, Pizer M, Rockman MV, Romano LA. 2003. The evolution of transcriptional regulation in eukaryotes. Mol Biol Evol 20:1377–1419.

Yost HJ. 1999. Diverse initiation in a conserved left-right pathway? Curr Opin Genet Dev 9:422–426.