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FROM MICROBIAL GENETICS TO MOLECULAR DARWINISM AND BEYOND

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When Charles Darwin reflected on the process of biological evolution some 150 years ago, he could not know of the existence of genes. But he and some of his contemporary natural scientists had observed that individual organisms belonging to a given species showed obvious phenotypical variations. Darwin's theory of evolution postulates that the different variants and their parents are steadily submitted to natural selection. This means that variants which can better deal with their encountered living conditions are favoured, and can, in the long term, overgrow less favoured organisms in the natural ecosystems.

Independently of evolutionary biology, Gregor Mendel initiated classical genetics in 1866, based on the observation that some phenotypical traits became transferred into the progeny and that recombinants could show up upon cross-fertility between individuals with different traits (i.e. mutants). The genes that were postulated to represent the determinants for phenotypical traits remained for many decades an abstract concept. This was still the case when around 1940 classical genetics and evolutionary biology joined forces in the so-called modern evolutionary synthesis that resulted in the Neo-Darwinism (Mayr, 1982).

About at the same time, microbiologists reported that bacteria and bacterial viruses could also undergo mutation and were thus postulated to possess genes. This opened the possibility to experimentally explore the formation of recombinants in mixed cultures of different bacterial or viral mutants.

Already in classical genetics there was good evidence that genes were associated with chromosomes. These were known to contain nucleic acids as well as proteins (chromatin). A strong evidence that desoxyribonucleic acid (DNA), rather than proteins, is the carrier of the postulated genes came in 1944, when Avery *et al*. (1944) reported their experiments with pneumo-

coccal bacteria. These authors worked with two different bacterial types that were distinct by their traits. DNA was extracted from one of the strains and carefully purified from all attached proteins. When this DNA fraction was incubated together with intact bacterial cells of the second strain, some bacteria showed up that had acquired the trait characteristic for the first bacterial strain: these bacteria had been transformed. No transformation could be obtained upon incubation of the second strain with the purified proteins of the first strain. DNA was thus concluded to be the carrier of genetic information.

How the genetic information can be inscribed in DNA became obvious several years later, when Watson and Crick (1953) showed that DNA molecules are long filaments with double-helical structure. Two antiparallel strands composed of four different nucleotides are held together by hydrogen bonds ensuring a specific base-pairing between the neighbour nucleotides. On the basis of this discovery it became obvious that genetic information can be contained in the linear sequences of nucleotides. In addition, correct base pairing was suggested as the criterium for the transmission of the genetic information from the parental genome to the two daughter genomes upon DNA replication.

In the following decades molecular genetics was developed thanks to experimental investigations with microorganisms and soon also with eukaryotic organisms. This then led to genomics including DNA sequence analysis and investigations on gene functions. The thereby acquired knowledge can provide insights into impacts that spontaneous alterations of nucleotide sequences can have on specific phenotypical traits. This kind of research turned out to be quite informative for an understanding of molecular mechanisms that generate genetic variations. The products of genetic variants together with their parental forms represent the substrate for natural selection. It is thus appropriate to join now forces between Neo-Darwinism and molecular genetics to result in Molecular Darwinism.

Bacterial genetics was developed with only a few kinds of bacterial strains, particularly with *Escherichia coli*. Under laboratory conditions these bacteria propagate exponentially with a generation time of about 30 minutes between two cell divisions. Large populations can thus be obtained in one day. Since bacteria are haploid, having just one set of genetic information, spontaneously occurring mutants become phenotypically manifested quite fast. One can observe that, when a few hundred growing cells are reached, one new mutation shows up. With available research strategies the nature of newly isolated mutants can readily be analysed, both with

regard to the suffered DNA sequence alterations and with regard to their functional capacities under various growth conditions.

As more and more gene and genome sequences of various prokaryotic and eukaryotic organisms become available, sequence comparisons can give hints on the molecular mechanisms that might have been at the origin of the observed functional differences. Such comparisons are also welcome for bacterial strains that cannot be propagated under laboratory conditions. Still, it is of importance to base any conclusions from sequence comparisons on available knowledge on already identified molecular mechanisms that are at the source of newly generated mutants. Experimental data of such events can best be acquired in work with genetically well known bacteria and bacterial viruses.

It is good to mention here that in the relevant genetic literature two different definitions are used for the term 'mutation'. In classical genetics, a mutant displays an altered phenotype that becomes transmitted to the progeny. In molecular genetics, looking at DNA sequences, a mutation is usually defined by carrying an altered nucleotide sequence. In the meantime, we know that, as a rule, classically defined mutants have also an altered nucleotide sequence. But we also know that by far not all nucleotide sequence alterations lead to an altered phenotypical trait. Many novel sequence alterations are, often for known reasons, silent or neutral and have no immediate influence on life processes. On the other hand, there is a good consensus between researchers in the field that relatively few novel mutations are favourable and provide to the organism a selective advantage. Much more often new mutations are unfavourable. They can inhibit life processes to some degree or, in extreme cases, they can be lethal. These kinds of mutations provide a selective disadvantage. This situation gives us no evidence for a directive nature of spontaneous mutagenesis. In general, the spontaneous generation of genetic variants reflects some kind of randomness; it is not a directing response to an identified specific need. We will explain below that several specific molecular mechanisms contribute to the overall generation of genetic variations.

Figure 1 can guide us in the discussion on Molecular Darwinism. The top of the scheme shows the three pillars of biological evolution: Genetic variation as the driver of the evolutionary process, natural selection that directs evolution together with the available genetic variants, and geographic and reproductive isolations that modulate the process of evolution. The different living conditions and the effective size of the biosphere on our planet, as well as enzymatic repair processes, limit genetic diversity.

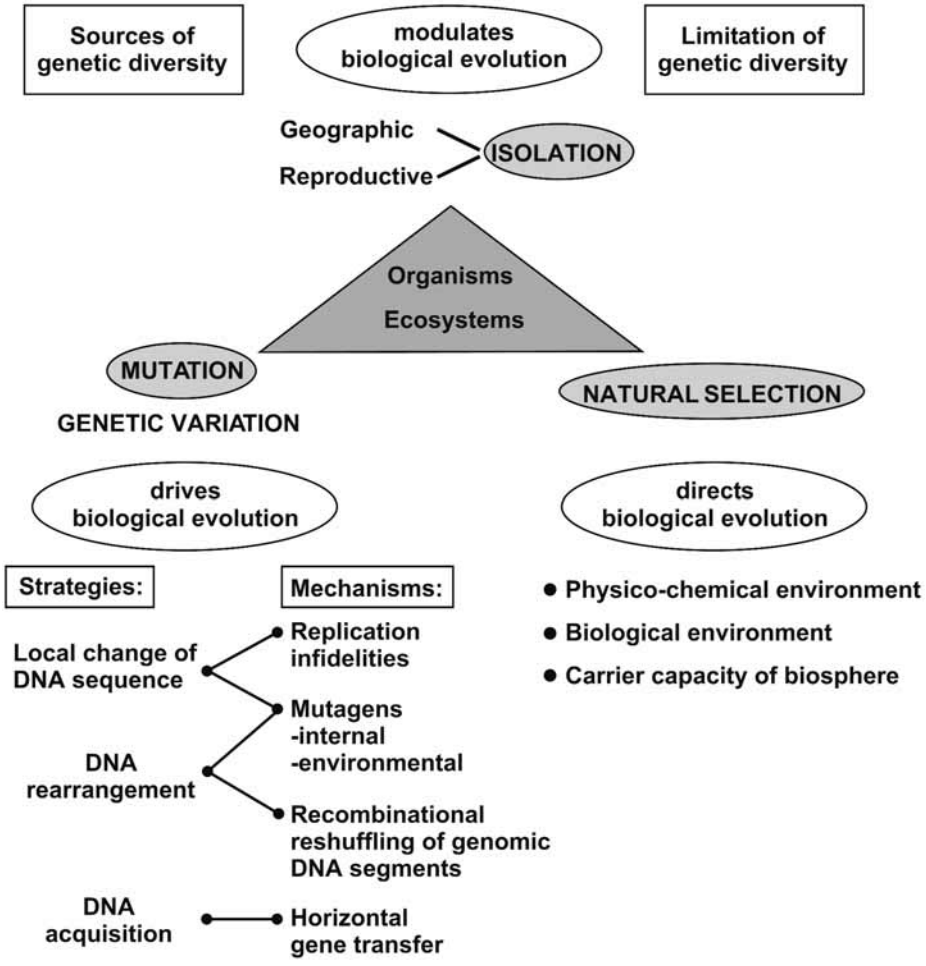

Figure 1. Schematic representation of elements involved in biological evolution and of the mechanisms and natural strategies of the generation of genetic variants (from Arber, 2008).

The lower part of Figure 1 shows on the left side in a condensed way the known sources of spontaneous mutagenesis. A detailed analysis to be commented below can lead to a classification of the different molecular mechanisms for the generation of genetic variation into three natural strategies of genetic variation: local sequence change, intragenomic DNA rearrangement, and DNA acquisition by horizontal gene transfer.

Before giving a more detailed explanation for these molecular mechanisms and strategies, it might be helpful for a better understanding to outline the main elements of the theory of molecular evolution (Arber, 2003, 2007) which is also called here Molecular Darwinism. We will see that a number of non-genetic elements contribute in reality to genetic variation. These elements are to a large extent intrinsic properties of matter, such as a certain degree of chemical instability of nucleotides. Another of these intrinsic properties relates to structural flexibilities of biological macromolecules, such as isomeric forms, in particular of nucleotides. Their tautomeric forms affect the proper base pairing in the double-stranded DNA molecules. Environmental chemical and physical (radiations) mutagens contribute of course also to spontaneous mutagenesis. Some of these mutagens are internal metabolic products (Smith, 1992). Still another factor is random encounter, e.g. of a mutagen with a cellular DNA molecule or of a gene vector with a target cell upon horizontal gene transfer.

Detailed studies of genetic variation processes have revealed that quite often, specific gene products are involved in genetic variation. These products of so-called evolution genes can act directly as variation generators and/or as modulators of the rates of genetic variation. Some examples will be discussed below. This outline shows that natural reality takes actively care of biological evolution. Genetic variation should not be attributed to errors and to accidents occurring to the DNA.

Local DNA sequence changes include the substitution of a nucleotide by another nucleotide, the deletion or the insertion of a nucleotide and also a scrambling of a few neighbouring nucleotides. There is good evidence that some of these sequence alterations occur upon DNA replication. It is known that enzymatic repair systems can rapidly spot the onset of these kinds of replication infidelities (Radman and Wagner, 1986). Upon the so-called repair, at least some repair enzymes can distinguish between the parental DNA strand and the newly synthesized strand. Consequently, they use the parental DNA strand as a master to put the affected nucleotide sequence in the newly synthesized DNA strand back into the correct parental order. Although these repair processes are quite efficient, they do not work with a 100% accuracy. This provides to cell populations a few rare local sequence changes in some individual cells, on the one hand, and to the individuals in the cell populations a relatively high genetic stability, on the other hand.

Let us now focus our attention on intragenomic DNA rearrangements. Various recombination enzymes are known to contribute to this kind of genetic variations. Generally speaking, these recombination processes can

affect DNA segments of various lengths, often containing one to several genes, and they can lead to the duplication, the deletion, the inversion or the translocation of a DNA segment, depending on the specific activities of the particular recombination enzymes at work.

By speaking on genetic recombination, one usually thinks at the so-called general or homologous recombination. In this reaction, the enzymes bring together DNA segments of a high degree of nucleotide sequence homology. DNA strands are then cleaved and spliced together across the two partners. In higher organisms these reactions are exerted in meiosis, when recombinants between paternal and maternal chromosomes are produced. In contrast, they do not work in mitosis during the normal DNA replication before each cell division. Bacteria also possess enzyme systems for homologous recombination. Again, in normal cell growth the enzymes become not readily expressed. But when breaks in the DNA molecules appear, e.g. after high energy irradiation, the so-called SOS repair becomes induced which produces a relatively high level of enzymes for homologous recombination. As a consequence, survival rates after irradiation rise, since intact genomes can be reconstructed by homologous recombination between sister DNA molecules that are present as already replicated genomes before cell division.

Mobile genetic elements are widespread in living organisms (Shapiro, 1983). These are DNA segments carrying normally one to several genes. The products of some of these genes are enzymes called transposases. Their activities can promote a translocation of the element to other chromosomal locations, sometimes in conjunction with a duplication of the element. These translocations are usually called transposition. Most bacteria carry in their genomes such mobile genetic elements, some of which are called IS (for inserted sequences) elements. Well studied *E. coli* bacterial strains carry in their genomes several specific kinds of IS elements, mostly in several copies of each kind. Interestingly, practically each kind of IS element (IS1, IS2, etc.) follows its own functional criteria, both for the selection of novel insertion sites on the DNA molecules and for the control of the availability of transposase activities at a low level, so that rates of transposition are actually very low. For example, IS30 (Caspers *et al.,* 1984) becomes inserted most readily into a specific, relatively short nucleotide sequence, although at much lower rates it can also insert elsewhere (Stalder and Arber, 1989). In contrast, IS2 prefers to insert in particular DNA regions of a length of a few thousand base pairs (Sengstag and Arber, 1983). But within these regions insertion can occur practically anywhere; the used inser-

tion sites show no distinct sequence homology (Sengstag and Arber, 1987). Transposons are mobile genetic elements that carry a segment with ordinary chromosomal genes between flanking elements that are responsible for their ability to transpose.

Transposition is not limited to intragenomic translocation, it can also occur to plasmids and to viral genomes during their residence in the bacterial cell. The impact of these possibilities on horizontal gene transfer will be discussed below. In this context, it is important to mention that some viral genomes can be counted to mobile genetic elements: They can insert into the host genome and at some later time excise again. Retroviruses that are widespread in higher organisms are a good example.

These considerations lead us to discuss processes of site-specific recombination. Indeed, some viral insertions into chromosomal DNA are to a high degree site-specific, and so is IS30, as we have seen. An interesting kind of site-specific recombination has been described as the basis for so-called flip-flop systems that are present in some bacterial and in some bacteriophage genomes (Glasgow *et al.*, 1989). We refer here to a flip-flop system that promotes the periodic inversion of a DNA segment. This segment is flanked on both sides by a 26 base-pairs long consensus (relatively high homology) DNA sequence. These flanking consensus sequences are carried in inverted order. The enzyme DNA invertase brings together these consensus sequences. The DNA strands become then cut and alternatively religated in the middle of the consensus sequences. This process results in the inversion of the DNA sequences carried in between the two consensus sequences. Inversion occurs back and forth every few generations in a growing microbial culture. This kind of flip-flop provides means to have two different genome organisations in a population of microorganisms. A strong evolutionary impact of this DNA inversion system, however, becomes obvious by the experimental observation that the enzyme-mediated DNA inversion can sometimes also occur between a consensus sequence and another, so-called secondary inversion sequence. Many different such sequences have been identified (Iida and Hiestand-Nauer, 1987; Arber, 1995). These secondary inversion sites do not show distinct similarities to the consensus sequence, and their spontaneous use shows at most some statistical reproducibility. This fact points to a certain specificity of the interactions. By using secondary inversion sequences, site-specific DNA inversion represents a source for novel gene fusions and for the assembly of an open reading frame for protein synthesis with an alternative expression promoter signal. We can conclude that these activities can be consid-

ered as active generators of genetic variants of evolutionary relevance. Note that in contrast to the regular flip-flop activity, the rates of using a secondary DNA site for DNA inversion are very low. These enzyme systems are a source for evolutionary novelty and they respect genetic stability of most of the individual cells in which they are carried.

With these presentations of a few well studied enzymatically mediated systems to promote occasional intragenomic DNA rearrangements we are impressed of the rich diversity of natural possibilities to provide novel genetic variations. In all these cases, the resulting genetic variants are, of course, substrates for natural selection. As we have already discussed, a majority of resulting genome orders may be lethal or of disadvantage. But it is the minority of winners, of variants providing selective advantage, that count for biological evolution.

Let us now look at horizontal gene transfer and its impact on the natural evolutionary strategy of DNA acquisition. As we have already discussed, microbial genetics has contributed much to today's knowledge in this field. Microbial genetics had a rapid start in the 1940s. In the already discussed transformation, free DNA molecules can be taken up by receptor bacteria, either actively or passively, depending on the particular microbial strain involved. In bacterial conjugation (Lederberg, 1947), two bacterial cells that can belong to different strains, meat physically. DNA from the donor cell can thereby become transferred to the receptor cell. A so-called fertility plasmid acts thereby as a gene vector (Hayes, 1964). Besides its own transfer through the conjugation bridge, the fertility plasmid can also provide the transfer of parts of the genome of the donor cell. A third possibility for horizontal gene transfer is its mediation by some bacterial viruses serving as gene vectors (Zinder and Lederberg, 1952). Again, we realize that nature was quite inventive with regard to the specific molecular mechanisms (Arber, 1994). In some of the processes, recombinant DNA molecules between viral and bacterial genomes are incorporated into viral particles (specialized transduction); in other instances, it is just a DNA segment taken from the donor genome that becomes incorporated into a viral particle (generalized transduction). Horizontally transferred DNA segments contain sometimes mobile genetic elements such as a transposon. This can facilitate an eventual incorporation of transferred DNA sequences into the genome of the receptor cell.

None of the horizontal gene transfer processes is specifically oriented to particular receptor bacterial cells. Transfer depends generally on a random encounter. However, between such an encounter and a stable integration of

the foreign genetic information into the receptor genome, several barriers seriously reduce the chance of acquisition of foreign genetic information. First of all, there is a requirement for surface compatibility of the receptor cell. In transformation the foreign DNA must find its way into the cytoplasm of the receptor cell, either by an active or a passive uptake, as already mentioned. In conjugation, the two mating partners must provide means for the building of a mating bridge. In transduction, the viral gene vector must find on the bacterial surface receptor sites that are required for successful infection. Secondly, many bacterial strains are equipped with one or even several restriction/modification systems (Arber, 1965). Restriction enzymes provide efficient means to identify if incoming DNA is foreign or if it had been produced in the same kind of bacteria. In the first case, the penetrating DNA molecules are cut into fragments by the restriction endonuclease. Within a few minutes the fragments are then further digested by exonucleases. However, at low rates foreign DNA fragments escape full digestion and succeed to incorporate at least some of their genetic information into the genome of the receptor cell. Generally speaking, successful DNA acquisition occurs only rarely and mostly in small steps, involving a part of a gene or one to a few genes at once. More or less random acquisition of foreign genetic information can often disturb the functional harmony of the cell in question. This is then the last barrier acting against successful acquisition. The hybrid resulting from acquisition will often have a selective disadvantage, less frequently a hybrid may have an advantage. This then represents a positive step in the process of evolution.

After having discussed examples for each of the three natural strategies of genetic variation, we can now compare the qualities of contributions made by each strategy to the evolutionary progress.

Local sequence changes offer the possibility for steps of improvement of available biological functions. Theoretically, local sequence changes could also represent a source for an occasional new biological function. But this can probably only become effective when the function in question starts to represent a substrate for natural selection.

DNA rearrangements can be seen as a tinkering with available capacities (Jacob, 1981). Novel combinations of functional domains from different genes may, for example, lead to a novel biological function. On the other hand, DNA rearrangements can also provide an alternative expression control signal to a functional gene. Such genetic variants may then express either higher or lower quantities of the gene product in question, as compared to the parental forms.

Favourable acquisition of foreign genetic information can be seen as a sharing in successful developments made by other organisms. In successful cases, the acquisition (in one step) of a biological function that the receptor organism did not possess, represents an extremely efficient contribution to the evolutionary progress. DNA acquisition as well as intragenomic DNA rearrangements might sometimes be a possible explanation for a sudden emergence of novel properties in evolving organisms.

The theory of molecular evolution postulates that evolutionary fitness may be reached when organisms are genetically equipped with the capacity to profit from all three natural strategies to generate genetic variants. For each of these strategies at least one, or better a few, specific mechanisms should be available.

In the light of this request one can postulate that in the course of the long past periods of evolution, the evolution genes, i.e. the sources for variation generators and for modulators of the rates of genetic variation, may have become fine-tuned for their functional activities by second-order selection (Weber, 1996). This means that those populations of organisms which had reached a certain degree of evolutionary fitness, were in advantage for adapting to changing living conditions. This can explain why organisms that live today are actually able to evolve and, nevertheless, to provide a relatively good genetic stability to the individuals of evolving populations.

In the description of molecular mechanisms and strategies for the generation of genetic variants, we have mostly referred to microbial experimental evidence. However, there are good reasons to assume that this acquired knowledge applies also to higher organisms. In recent times, more and more evidence for this expectation becomes available, particularly from DNA sequence comparisons. In this context, one can mention that some genetic variation generators nowadays also serve at the somatic level. A striking example is found in the somatic assembly of functional genes for specific antibodies of our immune system. In addition, some repair systems taking care of limiting rates of mutagenesis carry out their functions also in somatic cells. As far as horizontal gene transfer is concerned, one knows that some animal viruses can serve as natural gene vectors. In addition, symbiotic cohabitation of various microorganisms in animals and in plants is a very likely source for occasional gene transfer in one or the other direction.

A classical representation of long-term biological evolution is the tree of evolution. This tree usually shows the vertical flux of genes from the stem of the tree through the branches up to their ends, representing today's organisms with their enormous diversity. By taking care of the concept of

the evolutionary role of DNA acquisition, we have introduced more or less randomly placed connectors between branches as symbols for horizontal gene transfer (Figure 2). While in the vertical flux of genes the entire genomes are involved, upon the horizontal flux of genetic information only relatively short DNA segments become acquired, as we have already discussed. As we can expect from this modified representation of the tree of evolution, living organisms are not only interdependent by common roots in their past evolution, they are also interdependent in view of potential contributions to their future evolutionary progress by horizontal gene transfer. This new knowledge merits to become part of our understanding of biological evolution and it can enrich our world view.

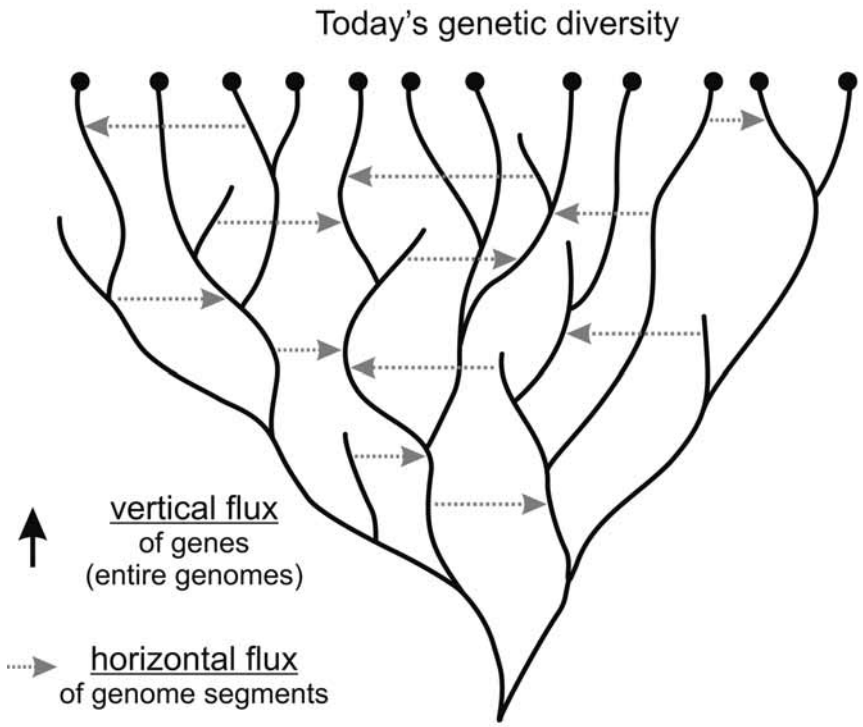

Figure 2. Actualized paradigmatic picture of the tree of biological evolution taking into account the evolutionary strategy of DNA acquisition by horizontal gene transfer (redrawn after Arber, 1991).

Another philosophical and world view aspect of molecular Darwinism is the notion of evolution genes. Although we know that some of their products serve also at the somatic level, other evolution gene products, such as at least some recombination enzymes and restriction/modification systems, clearly contribute only to the evolutionary progress of microorganisms. These genes are largely irrelevant for the bacterial life from one cell division to the next. We can thus conclude that genomes have a duality with regard to their content of genetic information. Many of their genes, such as housekeeping genes, accessory genes of use under particular life conditions, and in multicellular organisms the developmental genes serve for the fulfillment of the life of the organism. In contrast, the evolutionary genes ensure the capacity for biological evolution of the populations. Their products serve in cooperation with non-genetic elements for the expansion of life and for a slow, but steady, replenishment of biodiversity. Let me just mention that this philosophically interesting duality of the genome should not be taken as a strict classification of all the genes carried in the genome, since some gene products serve both for the needs of the individual life and for the capacity to evolve. However, the identified duality of the exerted functions can importantly contribute to a better understanding of the complexity of life and its evolution. From the scientific point of view, the living world of today reflects a long evolutionary path of permanent creation that may be based on a kind of self-organisation, and that must have its roots in the far past of the planetary evolution. The observed internal forces of the living world to undergo biological evolution gives us a guarantee that living organisms, at various stages of complexity, can continue to evolve and adapt to changing living conditions as long as such conditions will exist on our planet. Our actual knowledge on cosmic evolution predicts that appropriate conditions for organic life can still exist on our planet for about 5,000 million years.

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