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Essay

A pioneer of yeast genetics in Croatia: Zoran Zgaga's contribution to make national research acknowledged worldwide

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This article is dedicated to the living memory of
Professor Zoran Zgaga (1956–2011)

Calm sea skipper

Abstract

This study is an attempt to evaluate the pathway and the achievements of yeast genetics in Croatia. The study represents both, an authors' review and a historical overview and therefore is of value for yeast geneticists as well as for historians of science.

INTRODUCTION

Professor Zoran Zgaga, founder of Croatian yeast genetics, succumbed by heavy illness passed away at the age of 55 early last year. He was a distinguished lecturer and scientist both, in Croatia and abroad, and was a modest man known for his self-abnegation. In fact, his mild temper made his close friend, Professor Miroslav Radman, to say at the funeral: »Zoran was modest up to a point of anonymity« and also »Although I was the *older* one, nevertheless many times I felt like he was the *elder* one«. We believe that his loss will strike heavily Croatian genetics but we also believe that his legacy will continue to inspire many new generations of geneticists. Therefore, this article gives a short history of yeast genetics in Croatia in an attempt to acknowledge the scientific contribution of its founding father. Thus, this study is both, an author's review and a historical overview sporadically sprinkled by personal narratives. Reviews of general history of yeast genetics are available elsewhere (*1*, *2*). Other research groups from Croatia have chosen yeast *Saccharomyces cerevisiae* as a model system, but since their primal interest is not genetics, they are beyond the scope of this study.

Beginnings

At the time when Zoran Zgaga got interested in science the main model organism in molecular genetics was still *Escherichia coli*. Indeed, his initial scientific work was shortly centered on prokaryotes (*3*) but nevertheless his early post-prokaryotic period was steadily focusing on yeast *Saccharomyces cerevisiae* (*4*, *5*, *6*, *7*). It was only a decade after the first stable transformation of an eukaryotic cell (*8*, *9*, *10*, *11*) and little

Figure 1. *Zoran Zgaga, scientist vs. sailor. Science was Zgaga's life call but sailing was his breath-taking passion.* Bon vivant *as he was and if faced with a one-excludes-the-other choice we are (not) sure what he would have chosen…*

longer after the discovery that double-strand-break (DSB) was a powerful DNA damage capable of initiating recombination (*12*, *13*, *14*, *15*, *16*). The newly presented possibility to introduce exogenous DNA into a yeast cell opened the door to analyzing eukaryotic recombination on a molecular level and it was a big scientific sensation of that time. As a consequence, those pioneering works had promoted yeast as the main eukaryotic model organism of choice in molecular genetics for many years to come. Moreover, in 1983 the first model of DSB repair (DSBR) was presented (*17*) and the same authors also offered their views on the newly emerged transformation technology in yeast (*18*). Taking all that into account, it is reasonable to suggest that Zgaga's choice to switch to eukaryotic models was not random but rather enhanced by the contemporary scientific scene. Saying this we are actually picturing the pre-initial circumstances (and requirements) leading to (or necessary for) the development of yeast genetics in Croatia started upon Zgaga's return from his many scientific sojourns in France and the subsequent successful defense of his Ph.D. Thesis at the University of Zagreb more than 20 years ago. That historic fact has now become accepted in Croatian science.

However, before we systematically plunge into Zgaga's scientific legacy, it should be also noted that he had chosen to spend his post-doctoral research period in the USA collaborating with Michael A. Resnick (*19*). We cannot escape the impression that his choice was not accidental and must have had something to do with the fact that Mack Resnick was among the first to foresee the recombinogenic power of broken DNA (*12*). Since this is partly a historical study, it should be interesting to mention that such collaboration gave Zgaga an opportunity to communicate with Seymour Fogel (1919–1993), one of the pioneers of modern yeast genetics (*20*).

Early years

The early years of Zgaga's research were fully concentrated on the experiments accomplished within the scope of his Thesis (*21*) prepared under the mentorship of Professor Francis Fabre (Laboratory of Radiobiology, Institute Curie, Orsay, France). There, he was engaged in yeast co-transformation and inter-plasmid recombination, plasmid-chromosome recombination initiated by one base-pair (bp) mismatch situated on the plasmid, transformation with UV-irradiated plasmids including the role of several *RAD* genes in repair of UV-irradiated DNA, and the metabolism of short heterologous ends during homologous plasmid integration into the yeast genome.

Co-transformation and inter-plasmid recombination

One of the problems connected with yeast transformation that was poorly understood back then was the number of transforming molecules engulfed by the yeast spheroplast and their possible mutual interactions in the cell. In order to address those issues, an experimental system was developed to investigate inter-plasmid reciprocal recombination during co-transformation of replicative and non-replicative plasmids (*21*, *22*). A diploid strain was co-transformed by replicative and integrative plasmids (in 20-fold excess) sharing 80% sequence identity and transformants selected for integrative plasmid-borne marker were scored. Several co-transformation rounds were undertaken: co-transformation of replicative plasmid with either circular or linearized integrative plasmid cut within the plasmid homology (appropriate homology was either shared between the plasmids or present in the genome only) or in the plasmid backbone (no appropriate homology was present in the genome but it was shared between the plasmids). It was found that the fraction of cells receiving both plasmids was linearly increasing with increased DNA quantity and that recombination between circular replicative and non-replicative plasmids stimulated transformation efficacy by generating a joint vector. Thus, DSB introduced into the homology shared by both plasmids stimulated transformation efficacy 5-fold due to increased inter-plasmid recombination. Nevertheless, the stimulation was still 35-fold lower than DSB stimulation of targeted plasmid integration in the same system. However, if the DSB was introduced into the integrative plasmid region that shared no homology with the replicative plasmid, but shared homology with the chromosome, transformation efficacy decreased due to targeted recombination. Still, subse-

quent auxotrophical analysis of obtained transformants revealed that reciprocal recombination between co-transformed plasmids was 20-fold higher than plasmid-chromosome recombination during co-transformation. Demonstration that plasmid-borne DSB stimulated inter-plasmid recombination significantly better that the plasmid-chromosome recombination is interesting even today.

The nature of recombinogenic structures

The nature of recombinogenic structures generated upon mismatch repair of plasmid DNA was Zgaga's early research interest. Several early papers dealt with mismatch-stimulated recombination. One experiment revealed that a single plasmid-borne mismatch stimulates plasmid-chromosome recombination approximately 5-fold (*23*). Earlier, Fabre and Roman (*24*) had speculated that the cell recombination potential might be induced only by the uptake of irradiated DNA. In that case increased plasmid integration would have been a consequence of the increased recombination *per se* rather than of the plasmid-borne lesion repair. However, unpublished results reveal that the quantity of integrants was not increased by using irradiated carrier DNA (*21*). At that time, the idea that the repair of mismatches could lead to the formation of DSB that might then initiate recombination subsequently targeting plasmid integration was emerging (*25*, *26*). To address that possibility, a DSB was introduced into the plasmid bearing mismatch but further away from it. If during the repair of the mismatch a new DSB was to be formed the plasmid would have been inactivated lowering transformation efficacy. However, such effect was not observed (*23*). In an additional experiment, a UV-irradiated single-stranded (ss) replicative plasmid was used to transform yeast cells (*27*). UV significantly decreased yeast transformation efficacy by ssDNA showing that generated lesions lower its recombinogenic potential. In yet another experiment (*28*), it was revealed that UV-stimulated transformation in yeast requires damage on both plasmid strands increasing transformation efficacy by integrative plasmid up to 45-fold. Unlike double-stranded (ds) plasmids, and similar to ss-replicative plasmids (*27*), transformation potential of ss-integrative plasmids decreased upon UV-irradiation. Moreover, the proportion of transformants that arose by gene conversion increased 6-fold as a function of the UV dose.

Taken together, back then these experiments offered several conclusions. Although the possibility of DSB formation during mismatch repair was not indisputably ruled out, a view was proposed that mismatch-stimulated recombination is due to the formation of other structures, less recombinogenic that a broken DNA but more then an intact DNA. Stretches of ssDNA, appearing during repair process, were proposed as a type of recombinogenic structures apart from DSB (*23*). Results further suggested that in yeast, ssDNA is repaired interchangeably by both mutagenic and recombinogenic processes (*27*). Also, it was shown that UV increases yeast integrative transformation frequency with ds-plasmids by stimulating both, plasmid integration and gene conversion but decreases transformation frequency with ss-plasmids surprisingly leading to significant increase of gene conversion at the expense of plasmid integration (*28*).

It is interesting to speculate that the wide utilization of yeast replicative plasmid transformation in analyzing the repair of UV-induced DNA lesions (*29*, *30*) and the freshly developing possibility to achieve stabile yeast transformation with ssDNA at that time (*31*, *32*) must have had an impact on Zgaga's decision to use UV-irradiated ssDNA. Indeed, one of his discussion opening sentences was as follows (*27*): »The aim of this work was to introduce the use of yeast single-stranded replicative plasmids in the study of cellular repair processes operating on single-stranded DNA regions postulated to be the key structural intermediates in mutagenic and recombinogenic processes«.

Full sail ahead

Illegitimate recombination

Illegitimate recombination (IR) is very rare in yeast, unlike most organisms where non-homologous integration of exogenous DNA is much more common. We know now that the yeast cell has the potential to repair DSB by non-homologous end joining of broken DNA ends (*33*) but back then the possibility of IR in yeast was an exotic thought. At the time when Krešimir Gjuračić and Zgaga got attracted by yeast IR, there had been very few studies covering the subject. Nevertheless, two classes of illegitimate events in yeast, (*i*) restriction enzyme-mediated events where integration of exogenous DNA is mediated by *in vivo* action of a restriction enzyme, and (*ii*) IR events where integration of transforming DNA is effected by topoisomerase I, had been already reported (*34*, *35*, *36*, *37*).

Unlike the contemporary studies on yeast IR, which were conducted by dsDNA, Zgaga's study additionally included ssDNA making an important difference in main questions addressed at that time. His considerable earlier experience with ssDNA-transformation in yeast (*27*, *28*) must have been the foundation through which deeper insight into yeast recombination mechanisms was achieved later on. In one experiment, a yeast strain deleted for *ARG4* gene was transformed by circular or linearized ss-plasmid containing the *ARG4* gene and thus aborting any possibility of obtaining stabile Arg^+ transformants except by illegitimate integration (*38*). It was found that illegitimate integration efficacy of both, circular and linearized ss-plasmids was higher for up to 400-fold in comparison to their ds-counterparts. Molecular analysis of the integrants revealed numerous peculiarities of yeast IR. More than half or two-thirds of the integrants obtained by ss-circular or ss-linearized DNA, respectively, had the plasmid integrated into chromosomes and the rest had mainly arisen by formation of autonomously replicating elements. Also, it was shown that multiple tandem integration was frequent and stabile associated event.

Since the experiments had clearly demonstrated that IR could lead to significant genome rearrangements it had been important to see whether some lethal events for haploid cell could be rescued by transforming a diploid cell. Comparison of transformation efficiency by ssDNA in diploids and haploids showed increased number of transformants in diploids when transforming by both, circular and linearized ss-plasmid.

This experiment showed that yeast IR is very efficient when no extensive homology is present in the genome. However, it was perhaps more important to see its efficacy when appropriate homology was shared between the plasmid and the genome. In a separate research, a unique experimental system was designed by K. Gjuračić and even now some of us cannot escape recalling Zgaga's enthusiastic encomiums. Through this simple system it was possible to differ whether plasmid integration happened through targeted or illegitimate integration (*39*; Figure 2). This experiment clearly demonstrated that IR in yeast is possible even in the presence of extensive genomic homology. However, compared to the homologous integration in the same system, IR was up to 1600-flod less efficient.

Giving the extensive power of IR to rearrange the genome (*35*,*38*) next it was interesting to compare the sporulation abilities between the illegitimate integrants and the replicative tranformants. Decreased sporulation ability and sporadically spore viability had been demonstrated for the illegitimate integrants (*40*).

These experiments reached several conclusions: (*i*) IR in yeast is possible even in the presence of extensive genomic homology; (*ii*) ssDNA is a better substrate for IR in yeast; (*iii*) the yeast diploid cell can tolerate some genetic alternations that would be lethal for haploids; and (*iv*) IR in yeast leads to decreased sporulation efficacy and spore viability. These experiments had given Zgaga and his coworker an opportunity to look deeper into yeast recombination mechanisms as well as into yeast genome evolution. The decision to use ssDNA not only let them use yeast transformation as a model for studying recombination but also foresee the use of ssDNA transformation as a model for genome evolution.

The first possibility was ratified already with the proposed, although not shown by a figure, model (*38*). We are now prone to graphically show the model proposed back then (Figure 3). Briefly, the plasmid molecule could become »assimilated« into the newly synthesized strand during replication by priming DNA synthesis at the 3'-end and being ligated to the growing strand at the 5'-end. Chromosomal rearrangements could arise if ssDNA ends associate with two different genomic targets.

As for the second possibility, experiments showed that the integration of ssDNA in yeast could have important consequences on genome stability. Since it had been long known that the wild yeast cell is resistant to ssDNA transformation (*41*), this study had suggested that the barrier to ssDNA uptake in yeast is due to an evolutionary adaptation limiting the possibilities of horizontal gene transfer and thus increasing genome stability (*38*). Furthermore, it had been suggested that the well known low sporulation efficacy and spore viability in industrial yeast strains might be also facilitated by spontaneous illegi-

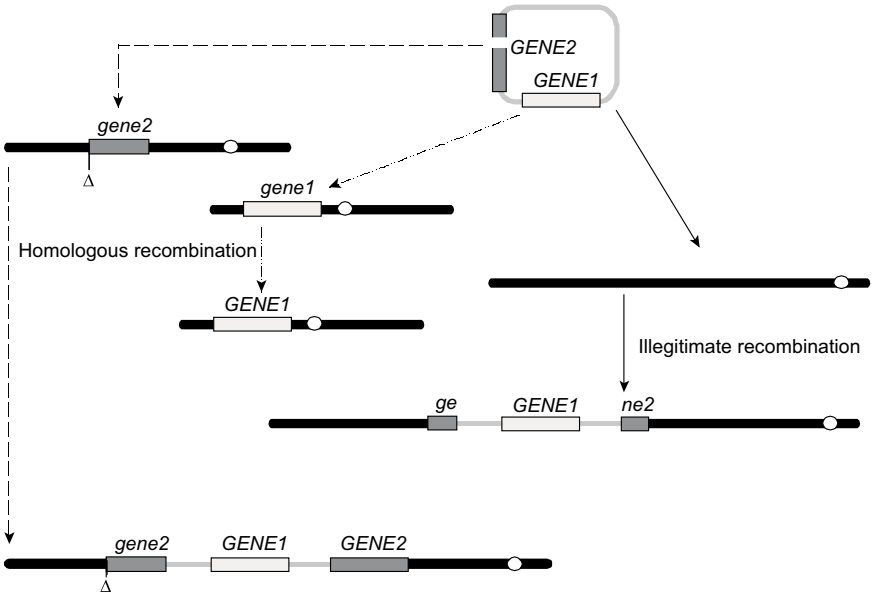

Figure 2. *Experimental system designed to differ between targeted and illegitimate integration in yeast (39). Plasmid molecule bears two selectable markers (yeast homologies) and prior to transformation is linearized within one of them. Appropriate genomic homology is promoterless and targeted integration restores its functionality thus leading to transformants prototrophic for both plasmid markers. In case of illegitimate integration, transformats are becoming prototrophs only for the plasmid homology (marker) that bore no initial break. However, targeted recombination without restoration or conversion of second genomic homology would also lead to single marker prototrophs. Therefore, all single marker prototrophs need to be Southern-blot checked. Δ designates the promoterless genomic homology. White circle represents the centromere.*

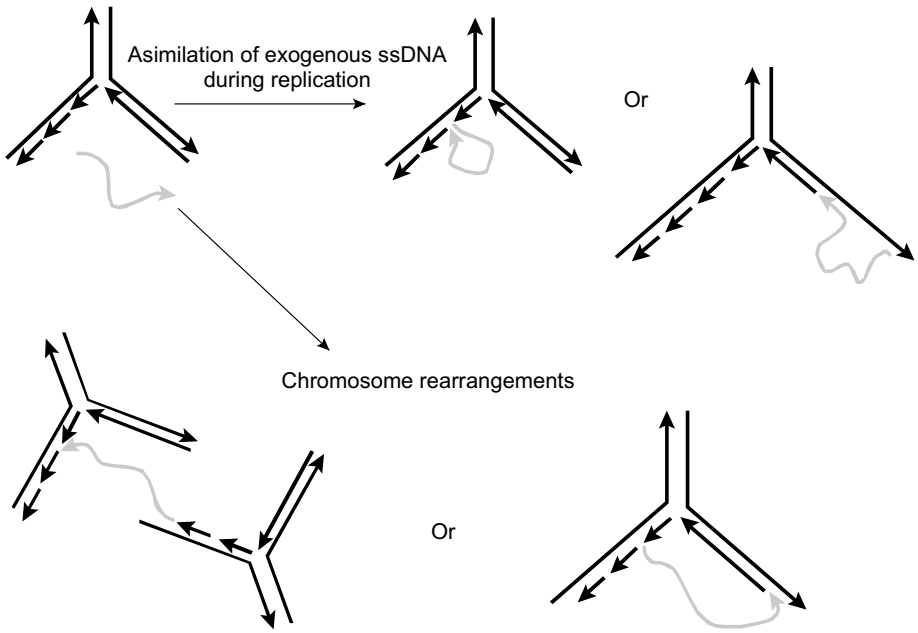

Figure 3. *Model of illegitimate recombination in yeast (38). Black: chromosomal DNA; grey: single-stranded exogenous DNA. The arrow represents 3'-end.*

timate recombination events after prolonged vegetative growth (*40*). On the other hand, in natural yeasts possible genomic rearrangements after illegitimate recombination could be stabilized in homozygous diploids through sporulation-based process of genome renewal (*42*).

Homologous recombination

Zgaga's deep interest in yeast recombination mechanisms has profoundly enriched Croatian general genetics by opening additional fascinating sub-field(s) of research. His interest in yeast homologous recombination (HR) mechanisms was so tenable that one of his late projects simply had to be titled »Mechanisms of recombination in yeast«. Although it may seem that such a general title might have concealed the concrete research goals, we would like to believe that it was his fascination with the field that led him to chose it. Indeed, as we can see in this section, numerous topics of yeast molecular genetics were covered by this project leading to several promising conclusions. Moreover, with this round of investigation Zgaga had managed to set a scene that later on let him and his coworkers propose even bolder con-

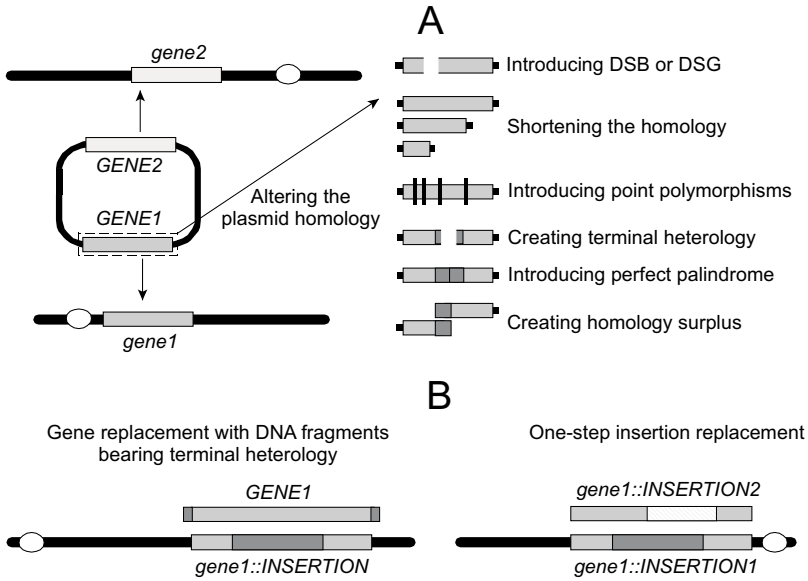

Figure 4. *Ends-in and ends-out assays used to analyze yeast homologous recombination in our Laboratory (39, 47, 48, 49, 50, 51, 52, 53). A. Ends-in assays; B. Ends-out assays. DSB: double strand break; DSG: double strand gap. Vertical black bars represent point polymorphisms. White circle represents the centromere.*

clusions. In this section we will try to briefly present the main experimental systems designed to analyze yeast HR in our Laboratory and to summarize the most important conclusions gained by those studies. Numerous mechanisms and pathways of HR in yeast are known today and their genetic and biochemical features and mutual interrelations are generally summarized elsewhere (*43*, *44*, *45*, *46*).

Experimental system(s) (ends-in vs. ends-out assays). A single ends-in experimental system was altered to better accommodate our research interests (Figure 4,A) (*39*, *47*, *48*, *49*, *50*, *51*, *52*). There, non-replicative plasmid bearing two yeast homologies was used to transform competent yeast cells. Prior to transformation, plasmid homology was altered and thus the influence on yeast HR was analyzed. Also, different experimental system(s) were designed in order to study yeast HR achieved through ends-out mechanism(s) (Figure 4,B) (*50*, *53*).

Substrate requirements. HR may be looked upon as a biochemical reaction where two DNA molecules transfer and/or exchange bits and/or chunks of strands through enzymatic action(s). Thus, it was potentially important to uncover the HR substrate requirements hoping that such insight would help better understand its mechanisms. Accordingly, Zgaga with his coworkers had been systematically addressing the influence of sequence polymorphism (*47*), homology length (*47*), heterologous DSB--ends (*50*), and large heterology within recombining homology (*52*) on mechanisms of HR in yeast.

The frequency of recombination between a pair of distant markers is generally proportional to the physical distance separating them (*54*). Decades later, this has opened a question of what is the correlation between genetic and physical lengths. Subsequent experiments revealed that there is a homology threshold, *minimal efficient processing segment* (MEPS), below which the recombination becomes significantly inefficient (*55*). In yeast, mitotic MEPS was estimated on 250 bp (*56*) and meiotic between 150–250 bp for reciprocal exchange (*57*). DSB induced recombination between repeated sequences needs MEPS between 63–89 bp (*58*) but only 26 or 30 bp of homology is sufficient for interplasmid gene conversion or gap repair of exogenous DNA, respectively (*59*,*60*). Thus, in yeast different recombination events require different MEPS. In one of Predrag Koren's experiments (*47*), plasmid homology was gradually shortened and targeted integrants were scored after circular plasmid transformation. Size-dependant recombination efficacy decrease was observed between 858 and 363 bp of homology during plasmid integration (*47*). However, homologies of 321, 259 and 107 bp had fixed plasmid integration value (2%) even though the homology length dropped up to 3-fold (*47*).

Over evolutionary time, genetic material accumulates polymorphisms posing influence on HR between diverged sequences even within the boundaries of the species. Earlier, it was shown that sequence polymorphisms may decrease or even abort recombination between diverged sequences and it was proposed that the phenomenon could lead to genetic isolation and thus eventually to speciation (*61*,*62*). This question is potentially interesting for both, geneticists and evolutionists interested in speciation. It is important to understand the cell mechanisms leading to recombination rejection and to see their possible correlation to speciation. Accordingly, models exist to explain the recombination rejection mechanisms in yeast (*63*). In our experiments, plasmid homology was *in vitro* mutated to integrate from one to six point mutations and targeted integrants were scored after circular plasmid transformation. Thus, the influence of a low polymorphism within circular ds- or ss-plasmid homology on HR efficacy during targeted integration was studied. The specificity of the system was in testing the influence of low heterology, usually existing between species or populations, on HR in yeast (*47*). The polymorphism of only 0.1% decreased the integration proportion for more than 2-fold. However, 6-fold higher polymorphisms decreased the integration frequency only 3-fold suggesting that additional mutations do not have cumulative effect for ds-plasmids. The presence of two or six point mutations did not influence homologous integration of the ss-plasmid either. It is interesting that up to one-fifth of integrants were obtained due to illegitimate recombination once again suggesting that ssDNA might be a better substrate for recombination even in the presence of homology (*38*, *39*). Finally, these experiments gave further evidence that ssDNA directly enters recombination without first being converted into dsDNA (*32*).

Taken together, this experiments leaded to several conclusions: (*i*) homology of only 107 bp is sufficient for reciprocal recombination responsible for circular plasmid integration in yeast but size-dependant increase of targeted plasmid integration efficiency is expected only with 3-fold longer homology setting the corresponding MEPS value on more than 300 bp; (*ii*) reciprocal recombination between ds-circular plasmid and yeast chromosome is affected by very low sequence divergence but no cumulative effect was detected supporting the view that sequence divergence does not suppress initial hybrid formation but rather some subsequent recombination steps; and (*iii*) low polymorphism within ss-plasmid had no effect on recombination efficacy suggesting that ssDNA might be less sensitive to sequence divergence. Therefore, these conclusions offered a view that the influence of homology size and polymorphism might be more complex that had been previously reported. Moreover, Zgaga and his coworkers were allowed to discus several interesting ideas. A mechanism spanning the size-dependant HR and IR in yeast was foreseen and loading of mismatch recognition proteins during the later recombination steps, possibly after the formation of Holliday junctions, was scrutinized. Also, it was argued that the exponential decrease in recombination frequency leading to reproductive isolation might become operational only after the polymorphism typically found within a species (<1%) is exceeded.

During the initial phase after the DSB appearance, the yeast cell provokes degradation of its 5'-ends leaving 3'-extensions capable of initiating HR in order to accurately repair the broken molecule. This alludes that the 3'-ends must be predominantly homologous to the target in order to be capable of finding the appropriate homology and/or to be apt to initiate DNA synthesis being a substantial part of the repair itself. Thus, the presence of terminal heterology is an important question that has been systematically addressed by several research groups. Experiments on the influence of terminal heterology length on DSBR with replicative plasmid assays (*64*, *65*, *66*) have revealed that the presence of heterologous ends had only minor influence on DSBR efficacy in wild type (wt) strain. On the other side, in *rad1*, *msh2*, *msh3*, *srs2* and *pol3-01* backgrounds, DSBR was severely impaired (*65*). However, in order to analyze the terminal heterology influence on DSBR, Zgaga had earlier used the non-replicative plasmid transformation approach and a few initial conclusions were reached (*21*). Using essentially the same experimental system, his coworker Ivan-Krešimir Svetec years later has managed to systematize and considerably broaden the initial study. There, the transforming plasmid contained a 102 bp multiple cloning site (MCS) within the plasmid homology and inside-cutting by different enzymes leaded to the linearization of the molecule simultaneously leaving terminal heterology of controlled length. Targeted integrants were scored upon transformation and relative transformation efficacy was calculated with respect to same linearized DNA bearing no terminal heterologies. The terminal heterology length was ranging from 0 to 46 nucleotides protruding from the 3'-end. Moreover, in one case the plasmid contained a head-to-head dimer of MCS and in another one copy of the MCS was replaced by a synthetic polylinker (*50*). Therefore, this excellent experimental system offered a possibility of analyzing the influence of terminal heterology on DSBR when (*i*) being identical on both ends, (*ii*) different on each end, and (*iii*) present only on one end (one-sided). Yet, Zgaga was interested in analyzing the effects of terminal heterology when DSB-ends were pointed outward and ends-out approach was subsequently developed by his coworker Anamarija Štafa (*50*). Such experimental approach allowed Zgaga's group to directly compare the transformation efficiencies of both recombination systems. Finally, numerous integrants were molecularly analyzed in order to screen the recombination events resulting from HR in the presence of terminal heterology.

Results have revealed that a short terminal heterology, of approximately 30 bp, significantly decreases yeast HR efficiency regardless of whether DNA-ends are pointed inwards or outwards. However, when the terminal heterology was present only one-sidedly the integration efficacy dropped by approximately half, again regardless of where DNA-ends were pointed. Comparison of ends-in *vs.* ends-out transformation efficiencies showed that exogenous DNA bearing inwardly pointed ends transforms yeast cells 100-fold more successfully. Molecular analysis revealed that transformants rose by HR (mainly) but also by IR and miscellaneous ends-in or ends-out characteristic recombination events emerged. During ends-out recombination even one-fourth of the transformants rose by IR in comparison to only 1% found in ends-in assay. However, targeted integration during ends-in recombination was not always accompanied by complete terminal heterology loss and up to 20% of the transformants had it integrated into the genome. On the contrary, during ends-out recombination two-thirds of the transformants rose by simple allele replacement during which terminal heterology was completely lost and one-fifth by integration into downstream part of the targeted genomic homology during which the terminal heterology was preserved and integrated into the genome. A small part of transformants had doubled a chromosome during ends-out recombination. However, the most intriguing result suggested that IR might be up to 5-fold more frequent with the appropriate targeting homology present in the genome.

Taken together these results suggested several important conclusions: (*i*) yeast HR is significantly impaired only when one of the recombining partners bears terminal heterology on both ends suggesting that stable intermediate formation might be achieved even when the other end is not entirely homologous to the target; (*ii*) the fact that the size-dependant curve shows sharp decrease only after reaching certain terminal heterology length possibly points to two mechanisms involved in its elimination during yeast HR; (*iii*) extensive terminal heterology degradation exists either prior to or along with the integration into the genome and cases of genome incorporated heterology could be outcomes of homology-associated non-homologous end-joining regardless to where DNA ends are pointed; (*iv*) ends-out recombination might be a source of gross genetic rearrangements such as those seen in yeast deletion mutants (*67*); and (*v*) homologous interactions between the genomic homology and the transforming fragment might stimulate free-ssDNA liberation in the cell unexpectedly increasing IR over HR frequency even in the presence of extensive genomic homology (Figure 5).

The *ura3-52* is a natural yeast allele bearing a Ty1 insertion known for its low frequency of conversion leading to restoration of Ura-prototrophy (*68*). Interestin-

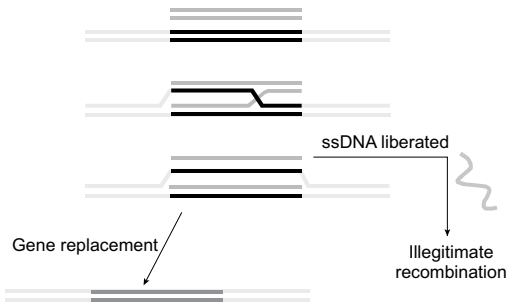

Figure 5. *Ends-out recombination as a source of illegitimate recombination (50).*

gly, the first successful conversion of a retrotransposone insertion during yeast transformation by exogenous DNA was reported by Zgaga's earlier group (*69*). Also, targeted integration bias characteristic for *ura3-52* was seen during yeast transformation by circular (*47*) and linearized DNA fragments with outward ends-orientation (*50*). Peculiarities of this yeast allele were constantly intriguing some of us during longstanding investigations of yeast recombination under the guidance of Zoran Zgaga. Some of the questions that had been systematically addressed were whether these peculiarities are characteristic solely to the Ty1 insertion or they rather associate to any large insertion and what are the molecular mechanisms allowing yeast cell to circumvent such potential obstacles to HR. Results have revealed interesting features of yeast HR between two substrates one of which is disrupted by a large insertion (*52*) and are being prepared for publishing elsewhere. Here, we can only express our sorrow that the study initiator is not around to see it published.

Palindromes. Palindromes are identical inverted repeats bearing no spacer sequences. Their sequential and/or conformational specificity have allowed them to be evolutionary selected as important DNA regulatory motifs but also have concealed a potential for creating genomic instability. Therefore, palindromes are of considerable scientific interest. They have been the longest and, along with terminal heterology experiments, the most fruitful research interest of Zoran Zgaga and his various groups. As some of us can recall, everything started with a simple experiment where a palindrome should have been inserted into a plasmid to be used for terminal heterology influence experiments. However, as one of us was about to found out soon, everything but easy was to clone a palindrome…

Here, we will summarize the most important results on palindromic influence to yeast HR that have been conceived and conducted in our Laboratory. In several substantial studies we were interested in seeing whether palindrome converts to DSB prior to initiating recombination (*48*), whether palindromes present in both recombining partners stimulate recombination (*49*) and what is the critical palindrome size to induce genetic instability in yeast (*51*). In one experiment, DNA was linearized prior to transformation and the ratio between the numbers of transformants obtained by the same linearized and circular plasmids was measured. Had the palindrome stimulated the recombination, change in the ratio would have been seen. However, results showed that 102 bp perfect palindrome did not stimulate HR when present only in the transforming homology (*48*). Molecular analysis of transformants showed that targeted integration frequency was lowered by a factor of almost 2 by the presence of the palindromic insertion in comparison to the case when non-palindromic insertion of comparable size was present instead (*48*). Earlier, it was proposed that DNA replication could lead to the formation of palindromic secondary structures that subsequently might initiate recombination (*70*, *71*). Since the plasmids used in the previous experiment were non-replicative, the observed lack of recombination stimulation could have been due to the inability of formation of secondary structures. Thus, it was next logical to see whether the recombination would have been stimulated if both recombining partners contained palindromes. Results revealed that in that case recombination was stimulated by a factor higher that 4 (*49*). Molecular analysis of the recombinants from two previous experiments showed interesting spectra of genetic events and among other had raised the question of what is the critical palindrome size to induce recombination.

In a separate study, an elegant experimental system was designed to quantify the influence of palindrome size on the recombination rate between two direct repeats (*51*). Increasing size palindromes were inserted in either one of two repeated genomic homologies or into the plasmid backbone between them (Figure 6). Control yeast strains contained a non-palindromic insertion in either one of the repeated homologies but not between them or contained no insertions at all. Recombination between the repeated homologies leaded to the deletion of the intervening plasmid sequence producing nutritional auxotrophs and pop-out rates were scored and compared. Results revealed that the recombination between two repeated homologies was stimulated more than 8-fold by the presence of a 110 bp palindrome situated between them in comparison to the configuration where the palindrome was absent. However, when the palindrome was present in either of the neighboring homologies, the recombination was stimulated only moderately and with non-palindromic insertion no stimulation was observed. Interestingly, in *sae2* background recombination stimulation was not observed for either, palindromic and non-palindromic configurations, leading to the conclusion that here palindrome-induced pop-out recombination was completely dependent on the *SAE2* gene. Finally, it was shown that a palindrome situated either in one of the repeated homologies or between them initiates recombination only after passing a certain critical homology size estimated on 70 bp. The longest palindrome used (150 pb) increased pop-out recombination frequency up to 20-fold.

Taken together, these results have reached several interesting conclusions: (*i*) palindromes are not efficiently processed to DSB in the yeast cell and DNA replication leading to palindromic secondary structures is needed to initiate recombination (*48*) as it was earlier proposed (*70*,

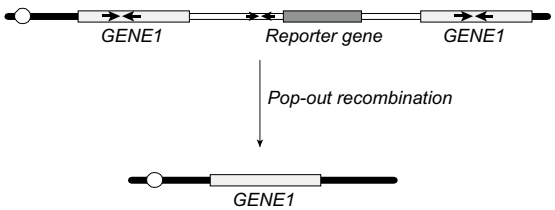

Figure 6. *Experimental system for* in vivo *palindrome-provoked recombination (51). Head-to-head arrows represent the locations of the palindrome. Black line: chromosome; White line: plasmid backbone; White circle: centromere.*

71); (*ii*) a significantly elevated recombination rate is possible only if palindromes are present on both recombining sequences, exogenous and endogenous (*49*) but a high pop-out deletion rate could be also accomplished when the palindrome is present in either one of the repeated endogenous homologies or between them (*51*); (*iii*) there is a threshold palindrome length capable of provoking size-dependant palindrome-induced recombination in yeast (*51*); (*iv*) *SAE2* might be critical for palindrome-induced recombination and since it is also needed for pop-out deletion (*72*) these results suggest that here deletions were generated mainly by single--strands annealing mechanism (*51*,*43*); and (*v*) long palindromes pose a source of genomic instability and, unless evolutionary preserved, should be eliminated by recombination.

Here it is also valuable to present some hidden aspects of this research. For instance, comprehensive molecular analysis of transformants was conducted. Zgaga strongly believed that thorough molecular analysis (if possible) is very important for acquiring a more complete picture and thus he wrote (*48*): »However, if transformation experiments are followed by molecular analysis of transformants, very fine, but biologically relevant effects can also be detected«. We would also like to acknowledge the experimental approach used to determine whether the palindrome converts to DSB prior to initiating recombination (*48*). The idea to use the ratio change between the numbers of transformants obtained by the same linearized and circular plasmids, of which the linearized one bears a palindrome, as a measure of targeting rate is intrinsically splendid.

Eukaryotic genome organization and mechanisms shaping it

Our understanding of the eukaryotic genome organization and possible evolutionary forces acting to shape it has recently progressed (*73*, *74*). Such growing new knowledge, among others, has uncovered that palindromes provide substantial genomic instability. Also, we are now able to distinguish between few palindrome-provoked mechanisms concealing potential to shatter genome tenability and thus to undermine cell existence and/or to shunt it towards tumorogenesis. In fact, some human disorders could be connected to palindrome-induced recombination (*75*, *76*). Therefore, palindromes are progressively alluring scientific awareness.

Although, Zgaga had earlier shortly denuded his thoughts on the utilization of yeast genetics to analyze the eukaryotic genome (*77*), years later, enthusiastically teasing his scientific imagination, palindromes awoke Zgaga's ambitions grossly. Soon, it was decided to prepare a catalog of all palindromes in the yeast genome and some of us still remember the exciting atmosphere in the Laboratory. A computer program was developed that was able to identify, locate and count palindromes within a DNA sequence in order to study palindrome content in the yeast genome (*78*). The palindrome counts in the actual yeast chromosome sequences were called *observed values* and were compared to median *expected values* gained from ten randomly computer generated yeast genomes. Results showed that observed values of each palindrome size were significantly different than expected values. Also, palindromes of 2–12 bp were under-represented in contrary to longer (>12 bp) ones that were over-represented, AT-rich and almost all intergenically located. Interesting, the longest palindrome contained 44 CG bp, suggesting active positive selection. Finally, it was discovered that the most under-represented dinucleotides were situated at the center of longer palindromic sequences.

Several general conclusions could be extracted from this work: (*i*) the obvious distinction between short and long palindromes, with the threshold situated at 10–12 bp, is very likely evolutionary shaped; (*ii*) positive evolutionary selection may also accounts for the abundance of long palindromes and for their location and base composition preferences; (iii) known dinucleotide underrepresentation was previously thought to either be due to codon bias (*79*) or to mutagenesis and/or replication/repair bias (*80*) and this work clearly supports the latter.

These conclusions allowed Zgaga and his coworkers to offer few innovative and speculatively attractive proposals. Thus, avoidance of a phenomenon similar to RNA interfering was invoked in order to explain longer palindrome preference for intergenic regions. Also, the obvious AT richness of longer palindromes was discussed as possible evolutionary adaptation that mirrors their regulatory functions. Such integral view on yeast palindrome evolutionary dynamics was summarized by a model (Figure 7) where stabilization of short palindromes within the coding-regions is disfavored due to slight bias in mutagenic DNA replication. On the other hand, the longer AT-rich palindromes may further prolong their size only outside the coding regions due to slippage mechanisms subsequently adopting a function of regulatory motifs.

Although others have also dealt with yeast palindrome content (*81*, *82*), it is worth stressing that Zgaga and mainly his coworker Berislav Lisnić and others, have been the first research group to launch a fairly accurate estimation of palindrome content in an eukaryotic genome. That endeavor was soon recognized beyond the usual peer-review publishing when the *Saccharomyces* Genome Database at Stanford University decided to mark each palindrome along the physical map of the yeast genome (http://www.yeastgenome.org). It should be also noted that the Spinnaker program developed for purposes of this study has not been commercialized and is available at request.

Drifts and wanderings

Working with Zgaga, one might have fallen under the impression that he often restrained from reaching conclusions challenging the mainstream thinking. Some of us were even objurgating such resistance. However, we now incline to explain his boundedness by an intrinsic

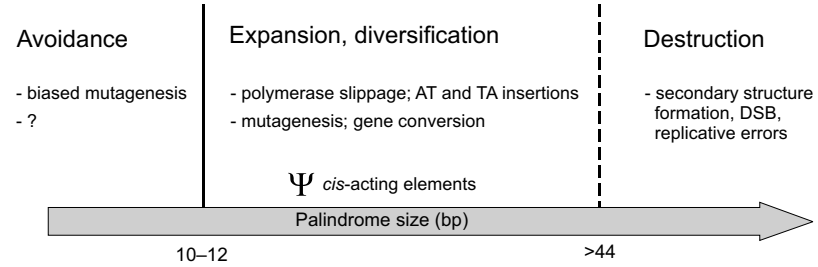

Figure 7. *Evolutionary palindrome dynamics in yeast (78; with permission). DSB: double strand break.*

desire to achieve delicate scientific accuracy while simultaneously leaving enough space for philosophical reverie. We also believe that this lack of vigor for unorthodox scientific thinking was only spurious since, as we can see here, mind drifting was not entirely strange to Zoran Zgaga.

Wonders of yeast transformation

Great applicable power, ordinary following basic genetical investigations, largely contributes to the development of modern industrial biotechnology (*83*). Much of those wonders are accomplishments of recombinant DNA technology (*84*). Accordingly, Croatian yeast genetics, perhaps due to being founded by a biotechnologist, have also offered a few applicable conclusions.

Yeast is today the main eukaryotic industrial organism with highly applicable transformation technology by replicative or non-replicative vectors. Moreover, non-replicative vectors have homed themselves as a powerful tool in basic genetics. They transform the yeast cell through recombination with its chromosome(s) during which process the exogenous molecule either gets integrated into the appropriate genome homology or donates genetic information to it (*85*, *32*). It was realized early on that the transformation efficacy with yeast non-replicative plasmids can be significantly influenced by *in vitro* modifications of the plasmid molecule. For instance, the introduction of DSB into the homology shared between the plasmid and the chromosome significantly stimulates the transformation ability of such linearized exogenous molecules. However, for Zgaga that was not sufficient. It was also interesting to see what are the others possible modifications that might influence the transformation ability of a non-replicative plasmid. As we stated before, his early group systematically studied the effects of plasmid conformation (ss or ds), plasmid linearization, sequence divergence between recombining partners, and UV-irradiation on transformation efficacy. Those results were bountifully summarized in two reports (*86*, *69*) and here, rather than commenting those unique results, we are prone to simply presenting them through Table 1.

A later work uncovered that yeast transformation efficacy could be further enhanced if *SGS1* and/or *EXO1* genes are inactivated (*87*). Thus, plasmid integration in *sgs1* or *exo1* backgrounds was stimulated up to 5-fold while double mutation showed synergistic effects stimulating the integration even up to 15-fold. This suggested that both genes are involved in different processes limiting targeted plasmid integration in yeast.

Taken together, the results on yeast transformation efficacy over the years have revealed several possibilities for biotechnological applications: (*i*) co-transformation experiments shed light on a possibility for *in vivo* construction of recombinant plasmids (*22*) in a similar manner as it had earlier been used for one-step gene replacement in yeast (*88*); (*ii*) UV-irradiation was proposed to be used as substitution for DSB when the restriction map is not known or suitable restriction enzymes are not available (*86*); (*iii*) ss-plasmids were seen as a powerful tool for introducing fragments of foreign DNA (*86*); (*iv*) DNA bearing short terminal heterology was proposed to be used for targeted genetic manipulation given sufficient transformation efficacy is provided especially applying for DNA fragments bearing terminal heterology one-sidedly (*50*); (*v*) inverted repeats were foreseen as encouraging the development of palindrome based integrative vectors (*49*); and (*vi*) since *SGS1* or *EXO1* homologues are phylogeneticaly frequent, their inactivation was proposed to enhance gene targeting in organisms with poorly developed transformation technology (*87*). Proposing these applicative conclusions, Zgaga must have been fully aware that in the future biotechnology would rely on a better understanding of the eukaryotic genome. Indeed, he wrote (*77*): »Further progress in genetic manipulation of eukaryotes will depend largely on our understanding of the structure, organization and dynamics of eukaryotic genomes«.

Homology surplus, ends-out tandem integration and multi-partner recombination

Unlike in meiosis, HR during mitosis sparsely completes by producing reciprocal progeny (*43*). However, some experiments seriously challenge this assumption. Thus, equal probabilities of repair by both, reciprocal or non-reciprocal recombination, were initially anticipated leading to the conclusion that recombination with the chromosome occurs with equal probability either with or without plasmid integration (*89*). Allotment of reciprocal events might depend on the recombining homologies locations and/or their nature (*90*). High proportion was observed when either both (*91*, *92*) or only one DNA homology (*89*) was situated on the plasmid. On the contrary, when both homologies were chromosome-borne reciprocal frequency was low (*43*). To address the

TABLE 1

The effects of different modifications introduced in yeast non-replicative plasmids on transformation efficiency and the type of the recombination event (86; with permission).

Plasmid modification	Transformation efficacy†		Recombination event
None	ds	1	Homologous integration Gene conversion
	ss	10–50	Homologous integration Gene conversion
Linearization within homology	ds	100–500	Targeted integration
	ss	10–50	Predominantly illegitimate integration
Linearization outside of homology	ds	0.1	Illegitimate integration
	ss	5–10	Illegitimate integration
UV-irradiation (200–1000 Jm^{-2})	ds	10–50	Homologous integration Gene conversion
	ss	1–10	Homologous integration Gene conversion increased
DNA-sequence divergence (0.1–0.7%)	ds	0.3–0.5	Homologous integration Gene conversion
	ss	10–50	Homologous integration Gene conversion Rare illegitimate integration

†Approximate transformation efficiency relative to native double-stranded plasmids; ds – double-stranded; ss – single-stranded

question of reciprocal exchange frequency in yeast, Zgaga's group used a replicative plasmid (*39*). The plasmid was linearized either by introducing DSB or DSG prior to transformation. To prevent DSB ends joining prior to inducing recombination, a *Δlig4* strain was transformed and stable or unstable transformants were observed. Stable transformants, bearing the homologously integrated plasmid, represented reciprocal exchange events while unstable represented non-reciprocal events. Results have uncovered that the observed low frequency of reciprocal exchange of only 3–7% (*39*) was in accordance with the previous assumption. However, when DSB recessive ends were filled in before transformation, generating a surplus of homology, the reciprocal exchange frequency increased up to 5-fold (*39*). Since the transformation screening was based on restoring the nutritional prototrophy, all the stable transformants had to first eliminate the mentioned homology surplus (prior or during plasmid integration) in order to restore the targeted chromosome homology. Thus, although an important departure from expected 1:1 ratio of reciprocal to non-reciprocal exchange for plasmid-chromosome recombination was observed, the frequency was in accordance with the general assumption for mitotic HR.

However, another study in our Laboratory utilized integrative plasmid targeting and reciprocal frequency close to 50% was observed in the wt strain (*52*). Thus, it was proposed that both recombination pathways, the conversion pathway and the synthesis-dependant strand annealing pathway (*43*), may account for the repair of exogenous DSB by HR in yeast (*39*). Recombination events that require additional steps, such as eliminating the redundant homology, may retard the recombination process leading to increased reciprocal exchange. Interestingly, in yet another experiment, high frequency of multiple plasmid integration was observed only in integrants containing terminal heterology incorporated into the genome (*50*). It was proposed that such recombination event requires longer time to accomplish the reaction allowing integration of additional plasmids to the same target. A separate idea leaded to a theoretical model where multiple plasmid integration was explained by simultaneous recombination of more than two DNA molecules (*52*). In that model, three or more exogenous DNA molecules compete for the same genomic target to which all are eventually paranemically positioned. After HR initiation by one DNA end from one molecule, the counterpart-ends from the other molecules compete for entering the reaction. Subsequent illegitimate joining of DNA ends from different molecules could lead to multiple plasmid integration. Previously, tandem integrations were explained either by integration of a single multimeric plasmid (*93*, *94*, *95*) or by sequential integration of separate plasmid molecules (*96*). However, the recent model provides a possibility of competitive targeted plasmid integration (*52*).

Multiple DNA fragment integration was also observed during ends-out recombination in yeast (*50*). There, homology-associated non-homologous end-joining was invoked to explain the phenomenon (Figure 8). Some of us still remember the hard time that I.K. Svetec had convincing Zgaga to publish the model. Perhaps, publishing the model eventually was the reason why he was later more prone to accept the model of competitive targeted plasmid integration.

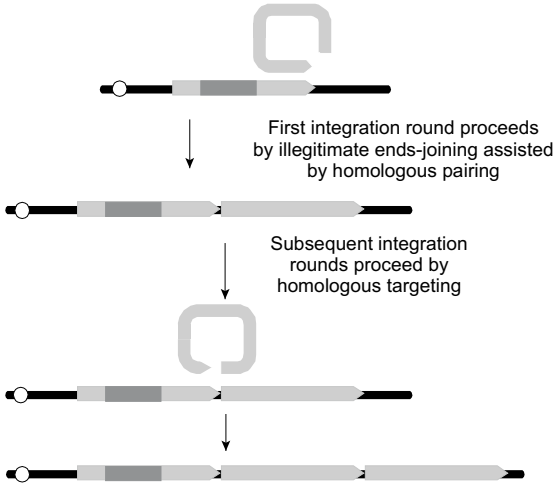

Figure 8. *Homology-associated non-homologous end-joining (50). Black line: chromosome; White circle: centromere.*

CONCLUSIONS

Croatian genetics has never been systematically evaluated although partial and/or personal narratives on its early years do exist (*97*, *98*, *99*, *100*, *101*, *102*, *103*, *104*). Therefore, this is an initial systematic evaluation of yeast molecular genetics in Croatia as a sub-field of general genetics. Entirely dependent on the exertions of a single man, Zoran Zgaga and his various research groups, yeast genetics in Croatia inevitably bears the stamp of his personal philosophy but also the restrictions that necessarily accompany such a unitary endeavor.

Early experiments in Croatian yeast genetics were concentrated on inter-plasmid recombination and the nature of recombinogenic structures showing accordance with the contemporary genetic trends of that time. Although the initial achievements were modest, nevertheless such pioneering work has laid foundation for future more sophisticated approaches where questions of deeper scientific essence were addressed. Work on illegitimate recombination was among the first investigations of that sort in worldwide yeast genetics. It solely has inspired important questions on genome evolution by horizontal gene transfer especially of single-stranded DNA substrates. However, the most regarded results of Croatian yeast genetics have been achieved in the field of homologous recombination and eukaryotic genome evolution. There, Zgaga with his coworkers fired from all weapons at disposal and struck precisely. It was soundly speculated that recombination impairment leading to reproductive isolation activates only after exceeding the polymorphism typical for the species. Ends-out recombination was depicted as a source of gross genetic rearrangements once again ascribed to the devastating power of single-stranded DNA only now, instead of exogenous origin, such harmful DNA was surprisingly unleashed during homologous recombination. Furthermore, for the first time a threshold palindrome length capable of provoking recombination in yeast was detected. Also for the first time, palindrome content in an eukaryotic genome was cataloged and an integral perception of their evolutionary dynamics was foreseen. Finally, several ideas of potential biotechnological interest were brought to day light.

Croatian yeast genetics has traveled a relatively short but objectively interesting path. In fact, we cannot clearly differ its possible declines from the upsurges. Therefore, the conclusion imposes itself that it is still growing and its zenith has not been reached yet. We have yet to observe how it will develop in the future when less biased writers would be in a position to evaluate it more accurately.

Epilog (Leveling the abandoned helm)

Almost 80 years ago Danish biologist Øjvind Winge (1886–1964) founded yeast genetics and started the yeast recombination fever (*105*, *106*). The fever has survived to witness recombination models rising and falling (*107*), the yeast cell transformed by exogenous DNA (*11*), even the whole yeast genome sequenced (*108*)… Zoran Zgaga got infected while enjoying the French cuisine and wine and passed it on to us. A little spark originating from the initial yeast recombination fever flame has traveled from Winge through Herschel L. Roman (1914–1989; visiting scientist in Winge's laboratory) and Francis Fabre (Roman's postdoc) to Zoran Zgaga (Faber's doctoral student) and finally it now enkindles us.

Acknowledgements: *We express our gratitude to Mrs. Sofia Blažević for correcting the English. This article was conceived and written by PTM.*

Addendum*: IKS is successor of Zoran Zgaga. KG is at ACIES BIO Ltd. in Ljubljana, Slovenia. PK is at Croatian Waters Co. BL has recently successfully defended his PhD Thesis and continues his work within the last Zgaga's group. MM is Zgaga's last doctoral student. MN is at Max-Planck--Institut für Experimentelle Medizin in Göttingen, Germany. AŠ is a postdoc at Lorraine S. Symington's Lab at Columbia University in New York, USA. PTM is spending his postdoctoral term within Krunoslav Brčić-Kostić's group at Ruđer Bošković Institute in Zagreb.*

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