OXFORD

The role of recognition error in the stability of green-beard genes

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Abstract

The empirical examples of the green-beard genes, once a conundrum of evolutionary biology, are accumulating, while theoretical analyses of this topic are occasional compared to those concerning (narrow-sense) kin selection. In particular, the recognition error of the green-beard effect that the cooperator fails to accurately recognize the other cooperators or defectors is readily found in numerous green-beard genes. To our knowledge, however, no model up to date has taken that effect into account. In this article, we investigated the effect of recognition error on the fitness of the green-beard gene. By employing theories of evolutionary games, our mathematical model predicts that the fitness of the green-beard gene is frequency dependent (frequency of the green-beard gene), which was corroborated by experiments performed with yeast *FLO1*. The experiment also shows that the cells with the green-beard gene (*FLO1*) are sturdier under severe stress. We conclude that the low recognition error among the cooperators, the higher reward of cooperation, and the higher cost of defection confer an advantage to the green-beard gene under certain conditions, confirmed by numerical simulation as well. Interestingly, we expect that the recognition error to the defectors may promote the cooperator fitness if the cooperator frequency is low and mutual defection is detrimental. Our ternary approach of mathematical analysis, experiments, and simulation lays the groundwork of the standard model for the green-beard gene that can be generalized to other species.

Keywords: green-beard gene, experimental evolution, sociality, evolution of cooperation, recognition error

Lay Summary

Cooperators should exclusively cooperate with cooperators while abandoning the defector. If not, defecting free riders will overrun, and cooperators will be extinct. Therefore, successful cooperators should accurately recognize the conspecific cooperators, which defines the characteristics of the "green-beard effect." Adhesion of the yeasts under external stress is a well-known example of the green-beard effect in that cells that secrete the adhesive materials aggregate. In this case, aggregation is cooperation. However, some cells that do not secrete adhesive materials are included in the aggregate (free riders), which can be considered as a recognition error. As no model until now analyzed this factor, we estimated how the recognition error influences the fitness of the green-beard gene. We theoretically predicted becoming a cooperator would confer much benefit if cooperator frequency is high and the mutual defection is punishing. In the case of yeasts, as defecting cells that do not secrete adhesive materials are less likely to be included in the aggregate, they would die if the external stress is severe. We confirmed these predictions through simulation and actual experiments. Interestingly, we speculate that cooperation with defectors might be advantageous to the cooperators if the cooperator frequency is low. Suppose that there are not many adhesive yeasts but a large aggregate is necessary to endure the stress. In this condition, establishing an aggregate with defectors rather than excluding them would be beneficial to the cooperators.

Introduction

It sounds axiomatic that genes that facilitate the spread of their own copies dominate the gene pool (Ågren, 2021; Dawkins, 1976; Gardner, 2016). One way to carry out such a strategy is to promote the fitness of the kin (or demote the fitness of the non-kin) as genealogically close kins have numerous genes that are identical by descent (kin selection *sensu* Queller 2011; see Hamilton, 1964; Malécot, 1948). As another strategy, suppose that there is a gene that enables the host to perform cooperative behavior. It is optimal for that gene to cooperate among those who have the same cooperative genes that are identical by state regardless of the relatedness (kind selection *sensu* Queller 2011, see Madgwick, 2020; Strassmann et al., 2011). The concept of kind selection was first formulated by Hamilton (1964) and later popularized by Dawkins (1976) who named the gene that can discriminate its kind the green-beard gene. Unlike kin selection, the kind selection was less attended to as it was considered to be implausible due to vulnerability to false beard, the gene with the appearance of the green beard, but without the behavior of the green beard (Grafen, 1990). In short, if there is any deceiving individual who takes advantage of cooperation from the possessors of the green-beard gene but defects to them, the frequency of the green-beard genes would plummet.

To distinguish the genuine sharer of the gene from the non-sharers, green-beard genes should have three inseparable traits by definition: (a) the genes should exhibit perceivable yet distinguishing traits (inimitable signal); (b) the traits should be recognized by others who share the genes; and (c) the gene-sharers should act favorably to other gene-sharers compared to their activity toward the nongene-sharers (Dawkins, 1976). These traits enable the host to perform altruistic or cooperative behavior exclusively among the sharers but not with nonsharers. If these conditions are genetically separable (encoded by different genes), then the hosts with perceivable traits alone (false beards) will benefit from individuals with genuine green-beard genes. However, if a single gene (or tightly linked genes) can exhibit those three traits inseparably, then the green-beard gene is probable (Madgwick et al., 2019). Since the first discovery of the actual green-beard gene in red imported fire ants (Laurent & Ross, 1998), examples of green-beard genes are accumulating including the cases of myxobacteria (Pathak et al., 2013), social amoebas (Queller et al., 2003), and budding yeasts (Smukalla et al., 2008).

Flocculation (coagulation of small particles) of budding yeast *Saccharomyces cerevisiae* promotes cells to survive under stressful environments (Smukalla et al., 2008; Soares, 2011). For example, cells in the center of the floc are protected from chemical stress (lethal-level ethanol [EtOH] or Amphotericin B) while the isolated cells are often damaged or killed (Smukalla et al., 2008). In this sense, *FLO1* is facultative helping green-beard gene as the actor works differentially depending on the presence of the same gene at the recipient (facultative) and the formation of the floc increases the survival rate (helping) (Gardner & West, 2010).

The cell–cell adhesion in flocculation is primarily mediated by lectin-like Flo1 proteins that are clustered on the cell surface where the N-terminal domain of Flo1 binds to the mannose of other cells via Ca^{2+}-dependent, lectin-like interactions (El-Kirat-Chatel et al., 2015). As yeast cells without Flo1 has mannose on the surface, floc formed by Flo1 may include cells lacking Flo1 (Goossens et al., 2011). The cooperation of the individuals carrying the green-beard gene with the others lacking the gene can be considered as a recognition error.

Such recognition error is readily found in green-beard genes discovered up to date. Queens of red imported fire ants (*Solenopsis invicta*) with *BB* genotype at locus *Gp-9* were frequently attacked by workers with *Bb* genotype as they reached sexual maturity, while no *Bb* queen was attacked presumably due to odor cues (Laurent & Ross, 1998). In this case, *Gp-9ᵇ* is the green-beard allele (Laurent & Ross, 1998). In other words, *Bb* workers of red imported fire ants accurately recognize sharers (*Bb* queens) as sharers, while *Bb* workers often fail to recognize and attack the *BB* queens (nonsharers) presumably due to inaccurate recognition of chemical cues on the cuticular surface of the queens (Keller & Ross, 1998; Trible & Ross, 2016).

csA is a green-beard gene of social amoebas (*Dictyostelium discoideum*), which expresses cell adhesion protein on the cell membrane (Queller et al., 2003). When food availability becomes deficient, wild-type *csA⁺* amoebas form fruiting bodies while some of the cells making the stalk die altruistically. When mixed with *csA* knockout mutants, a certain portion of spores from the chimeric fruiting body included *csA* knockout mutants (Queller et al., 2003), reminiscent of the yeast floc composed of *FLO1⁺* and *flo1⁻* cells. Hence, cooperators (*csA⁺* amoebas) often fail to distinguish the defectors and thereby cooperate with them.

An intriguing empirical case of the green-beard gene with the recognition error is fusogenic *traA* of *Myxococcus xanthus*, which facilitates outer membrane exchange (OME) through which materials needed for survival are transferred (Pathak et al., 2013; Sah & Wall, 2020). *traA* alleles are highly polymorphic, and divergence of TraA variable domain blocks OME between the cells (Pathak et al., 2013; Sah & Wall, 2020). Hence, exclusive cooperation is guaranteed among the cells with identical *traA* types, satisfying the definitions of the green-beard gene.

Though homophilic interaction between the cells is required for the fusion of cell membranes, the functionality of chimeras and the existence of a specific residue for allorecognition (Pathak et al., 2013; Sah & Wall, 2020) hinder the accurate recognition of the green-beard gene. Interestingly, the addition of an intermediate strain facilitates the transfer of proteins between the two strains that cannot directly exchange materials due to the incompatibility of TraA (Sah & Wall, 2020). Namely, a pack of proteins could be transferred from one strain to the intermediate strain and then transferred from the intermediate strain to another strain (Sah & Wall, 2020). This indicates that protein transfer (cooperation) may occur between the cells that carry different types of green-beard alleles.

Furthermore, *traA* is not the only green-beard gene involved in OME. Analyzing OME in the evolutionary context is complicated due to another green-beard gene *sitA*, which entails the death of the recipient cells by selective toxic materials (polymorphic SitA lipoprotein toxins) (Vassallo et al., 2017), violating the basic assumption that the protein transfer is beneficial to the recipient. In this case, *sitA* is obligate harming gene, while *traA* is facultative helping green-beard gene (Gardner & West, 2010; Madgwick et al., 2019; Vassallo et al., 2017), and no model up to date has considered multiple green-beard genes located in a single organism (Madgwick et al., 2019). Multiple green-beard genes might be equivalent to the increased recognition error as it becomes difficult to recognize the genuine conspecifics (those who have the same alleles for multiple green-beard genes).

Examples of empirical green-beard genes indicate that there are certain error rates of recognizing the green-beard genes of others, letting defectors benefit from social cooperation. The invasion by defecting false beards was often used as the argument against the existence of green beards. These arguments assume that individuals have no capability to distinguish the genuine signals of the green-beard gene from those from the false beards. The ability to at least roughly distinguish the nonsharers confers the evolutionary robustness of the green-beard genes against the false beards. The standard model to analyze the stability of the green-beard gene hereafter should consider the recognition error, not confined to the benefit and cost of the actions.

In this article, we investigated how errors of recognition would affect the stability of green-beard genes against defectors in both theoretical and experimental frameworks. At the theoretical level, we first mathematically predicted the dynamics of the proposed system by employing payoff interaction games (Figure 1). To verify the mathematical findings in the experimental framework, we carried out both experimental tests by applying chemical stress to yeasts with or without *FLO1* and the numerical tests by employing spatial dynamics of the iterated games on square lattices. Along with the substantiation of previous findings, our mathematical analysis suggests that cooperation with defectors (by employing recognition error to the defectors) might be advantageous in certain conditions.

The framework of our interdisciplinary work is built upon the evolutionary game theory that is often used to explain the diverse
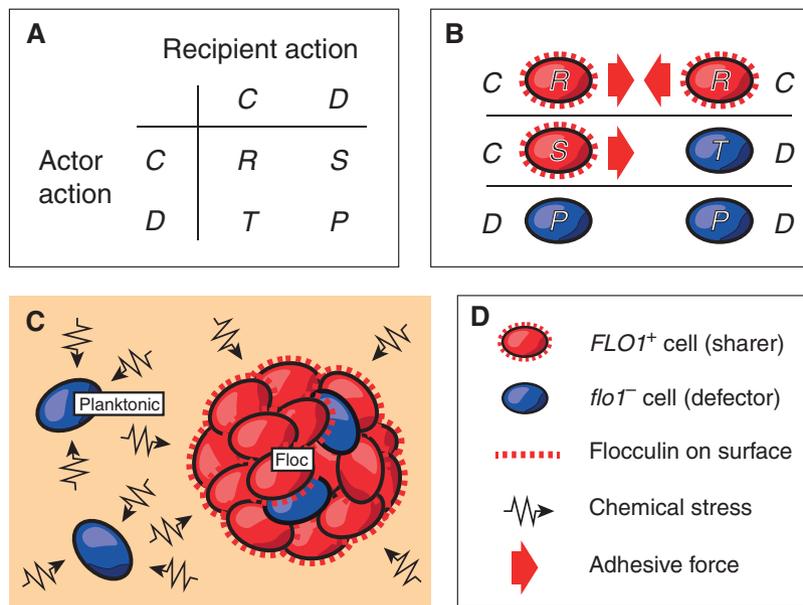
Figure 1. The general schematics of the game-theoretical interpretation of yeast green-beard effect. (A) The payoff table of interactions between the actor and the recipient. C refers to cooperation, and D refers to defection. R, T, S, P are the expected payoff of the actor determined by the actions of the actor and the recipient. (B) In an experimental sense, C corresponds to the provision of adhesion (mediated by secretion of flocculin illustrated by the spikes on the cell surface), while D corresponds to the nonprovision of adhesion. If there is no recognition error, cooperators ($FLO1^+$ cells) exclusively cooperate with the conspecific. Depending on which strategy the cell follows and the recognition errors, cells receive different payoffs (R, T, S, P), which later transform to fitness and the resultant allele frequency. (C) When exposed to chemical stress, the cells forming the floc (mostly $FLO1^+$ cells) are protected from the stress, while planktonic cells (mostly $flo1^-$ cells) are fully exposed to stress. However, secretion of the flocculin requires cost. These effects can be reflected in the payoff table and the baseline cost (Δ), making R, T, S, P the function against external stress and frequency of $FLO1^+$ cells. (D) The description of graphical components in (A–C).

aspects of social evolution by estimating the payoff of the strategic interactions (John Maynard Smith, 1982; Traulsen & Nowak, 2006). Recently, microorganisms, due to their modifiability and short generation span, have been used to prove evolutionary game theories experimentally (Gore et al., 2009; Kirkup & Riley, 2004; Lambert et al., 2014; Liao et al., 2019). By embracing the influence of the recognition error in the green-beard effect which has been less attended to, our framework provides practical insights into the evolutionary stability of the green-beard effect.

Methods

Strains

We used KV210 *S. cerevisiae* strain (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0, Galp-FLO1*) containing *FLO1* under GAL promotor (Smukalla et al., 2008) as the sharer cells (the cells with the green-beard gene). The defector cells (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, flo1Δ::HIS3*) were generated by substituting *FLO1* with an amplified HIS3MX6 cassette via homologous recombination using BY4741 strain (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Therefore, $flo1^-$ cells but not $FLO1^+$ cells survive on SC–His (synthetic complete medium without histidine) plates, while both types of cells can survive on SC (synthetic complete) plates.

Conditions for cell growth

Both types of cells were cultured in liquid YPD media overnight in the shaking incubator (30°C with 200 RPM). After washing the cells with dH_2O or YPGal by centrifugation, cells were grown in YPGal media for 2–3 hr (starting OD_{600} = 0.1–0.4) in the shaking incubator for the induction of *FLO1* under the GAL promotor. After the induction, cells were collected by centrifugation and diluted in YPGal, so that 1 mL of the suspension contains 0.01 g

of $FLO1^+$ or $flo1^-$ cells. We prepared 50-mL tubes and added the 0.02 g of yeast cells composed of sharers and defectors (starting mass ratio of sharers and defectors = 3:7, 5:5, 7:3). For example, to make the ratio of 3:7, 600 μL of YPGal media containing $FLO1^+$ cells (0.01 g/1 mL) and 1,400 μL of YPGal media containing $flo1^-$ cells were added. The media containing $FLO1^+$ cells were shaken before pipetting to maintain the homogeneity of cell concentration. Three conditions of chemical stress were tested: 0% EtOH (no stress), 10% EtOH, and 20% EtOH in 5 mL of YPGal media. After mixing two types of cells, they were vortexed for approximately 1 s to deflocculate the flocs temporarily. A set of three tubes containing different proportions of the cells under each stress condition was cultured for analysis. If any of the three tubes was improper to be analyzed (e.g., widespread contamination, too few or too many colony-forming units (CFUs) on plates; Chacón et al., 2018), all tubes were excluded from the analysis. Cells were grown in the shaking incubator with or without chemical stress for 2 hr, then deflocculated by the addition of EDTA. The media was consecutively diluted and spread on SC and SC–His plates. They were incubated at 30°C for 3–4 days.

Analysis

The CFUs on each plate (excluding the periphery of the plates) were manually counted with aid of AutoCellSeg (Khan et al., 2018). Based on this data, we deduced the proportion of sharer and defector cells after the stress exposure. Plates that exhibited uncountably many or unreliably too few CFUs were excluded from the analysis.

Take w_f and w_n as the Darwinian fitness (Crow & Kimura, 1970) of the flocculating and nonflocculating cells, respectively, which reflect the growth of the cell numbers in a given period. If p is the initial proportion of the $FLO1^+$ cells, then the proportion of

FLO1⁺ cells after the stress exposure would be $p^* = \frac{pw_f}{pw_f + (1-p)w_n}$. Take the relative fitness of *FLO1⁺* cells as $w_r = w_f/w_n$ (Orr, 2007), then $p^* = \frac{pw_r}{pw_r + (1-p)}$. Therefore, the following equation is derived (Kümmerli et al. 2009):

$$w_r = \frac{1-p}{p} \cdot \frac{p^*}{1-p^*}. \quad (1)$$

We used the generalized linear mixed-effects model (Bolker et al., 2009; Jiang & Nguyen, 2007) (MATLAB *fitglme*) to analyze the effect of the initial proportion and stress intensity with the logarithm link function.

$$\log(w_r) = \mu + \alpha_1 x_1 x_2 + u_b + \varepsilon \quad (2)$$

where α_1 is the estimate of the fixed effects, which is the multiplication of the initial proportion (x_1) and stress intensity (x_2), and u_b is the random effect caused by the set (cells from the same culture) in this model ($u_b \sim N(0, \sigma_b^2)$), while ε is the error term and μ is the intercept. The stress index was defined as the inverse of the average CFUs (scaled for tractability) of 5:5 proportion after 2-hr stress (Figure 2c). Stress intensity was assumed to interact with the initial proportion as the formation of the floc confers resistance to the stress, and flocs are formed when there are a sufficient number of *FLO1⁺* cells. In addition, the initial proportion is expected to exhibit less effect when the stress intensity is low. In Wilkinson notation (MathWorks, n.d.; Wilkinson & Rogers, 1973), it is represented as "$y \sim x_1{:}x_2 + (1 \mid S)$" with the log link function where y, x_1, x_2, S represent relative fitness (numeric), initial proportion (numeric), stress index (numeric), and set (categorical), respectively.

Simulation

In our simulation on a square lattice of size $N = 100^2$, an agent interacts with all of its von Neumann neighborhood (this neighborhood is defined as cells in the left, right, top, and bottom of the focal cell) by playing the games. We did not set the periodic boundary condition—a widely used condition for spatial systems of evolutionary dynamics (Hauert & Szabó, 2005)—for this simulation as yeast cells near the air–suspension surface or the wall of the experimental tube would face less interaction than other cells, which would be analogous to the agents at the rim of the lattice. Agents play games with all of their neighbors. (The number of interactions in 100 × 100 lattice = 19,800.) Through these iterated games whose payoff values are determined by Equations 5–7, each agent accumulates the payoff which later transforms into fitness. When games among all pairs are finished, the cost of being a cooperator (decreased overall payoff, Δ, in Equation 3) is imposed on each cooperator, which is analogous to the secretion of flocculin. Randomly chosen 20% of agents are eliminated and replaced by newly reproduced (replicated) agents ("death–birth" process; Nowak, 2006). Some agents of the remaining 80% reproduce, and the probability to reproduce is proportional to their respective fitness, determining the progeny frequency of the subsequent generation. This fitness-weighted reproduction is used in numerical simulations for cell growth or social behaviors (Lozano et al., 2020; Page & Nowak, 2001; Westy et al., 2016). After each round of the generation composed of iterated games to replication, all agents are spatially shuffled, resembling the suspension of yeast cells in the liquid media. The final results were measured after 20 generations (step-by-step description in Figure 3), and the average was obtained by 2,000 repetitions.

Results
Mathematical analysis

Suppose there are N individuals with two nonoverlapping characteristics: ones with green-beard genes and others with defecting characteristics. Throughout this study, N was assumed to be considerably large. The payoff matrix of interaction is shown in Figure 1, and suppose all values of the payoff matrix are non-negative for convenience. If there are i individuals with identical green-beard genes (henceforth, "sharers" in terms of genetics or "cooperators" in terms of behavior), then the number of defectors is $N - i$. Let β_S be the probability that sharer recognizes another sharer as the sharer (and acts accordingly to the recognition) and β_D, the probability that the sharer recognizes the defector as the defector, analogous to the type 1 and type 2 errors of statistical inference. We call β_S and β_D the accuracy of distinction to sharers and to defectors, respectively. The rule of the green-beard gene is that the sharer of the gene cooperates with the sharer (or the defector that the sharer think is a sharer by the recognition error), and the sharer defects to the defector (or the cooperator that the sharer think is defector). The defector always defects. In the case of yeasts, cooperation (adhesion) requires costs as they need to produce flocculin, which is represented as baseline cost,
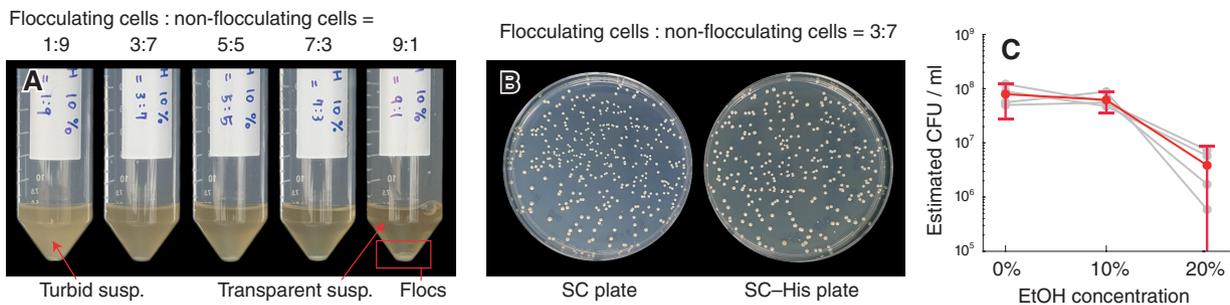

Figure 2. The process of stress exposure and spreading under 10%-ethanol stress. (A) The tubes containing yeast cells with different initial proportions in 10% (v/v) ethanol dissolved in YPGal after 2-hr stress exposure. Higher amounts of flocculating cells induced flocs in the suspension, by which the suspension became more transparent due to the lack of planktonic cells. Five rectified pictures were merged into one picture without any change in brightness or contrast. (B) After 2-hr stress exposure, cells were deflocculated by EDTA and equal amounts of diluted suspension were spread on SC and SC–His plates. Flocculating cells (*FLO1⁺* cells) cannot grow on SC–His plates. The number of colony-forming units (CFUs) was used to estimate the proportion of both types of cells after the stress exposure. The CFU counts of all plates used for analysis are shown in Supplementary Tables S2–S4. (C) The estimated numbers of CFU after 2-hr stress on SC plates with the initial ratio of 5:5 were used as the index of the stress in statistical analysis. Plates from the same culture were used for the analysis (the dots linked with light gray lines) to eliminate the effect of the viability of each cell culture. Red dots are averages, and error bars show 95% confidence intervals. CFUs under ethanol concentration of 0% or 10% do not differ much.
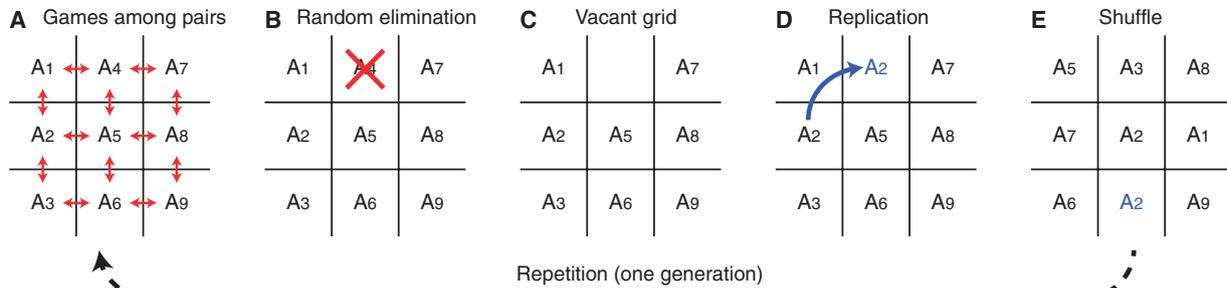
Figure 3. Schematics of the simulation steps for 3 × 3 exemplar lattice. (A) As the first step of a generation, each agent (A_1–A_9) plays games with all of its neighbors. The number of such pairs is 12 shown by red bidirectional arrows. Agents gain payoff values as a result of the iterated games. (B) Regardless of the accumulated payoff, the randomly selected agent is eliminated (A_4 in this case). (C) The grid of the eliminated agent becomes vacant, which later will be occupied by a replicated agent. (D) The accumulated payoff is transformed into fitness that stochastically determines which agent becomes the replicator (A_2 in this case). The higher the fitness of the agent is, the more likely the agent replicates. (E) The positions of the agents are shuffled, which is the last step of a single generation. Agents play games as in (A), initiating the subsequent generation.

Δ. How payoff values and recognition error should be biologically interpreted for the case of yeasts is described in Supplementary Material.

Based on these rules, the expected payoff of a sharer (π_i^S) and that of a defector (π_i^D) are

$$\pi_i^S = \tfrac{i}{N}(\beta_S^2 R + \beta_S(1-\beta_S)(S+T) + (1-\beta_S)^2 P) + \tfrac{N-i}{N}(\beta_D P + (1-\beta_D)S) - \Delta, (3)$$

$$\pi_i^D = \tfrac{i}{N}(\beta_D P + (1-\beta_D)T) + \tfrac{N-i}{N}P \quad (4)$$

by assuming $\frac{i}{N} = \frac{i-1}{N}, \frac{N-i-1}{N} = \frac{N-i}{N}$ for sufficiently large N. The case-by-case calculation of the expected payoff considering the recognition error is presented in Supplementary Table S1.

Once the payoff of the sharers and defectors are described, the frequency dynamics and fixation probability of each gene can be calculated by the Moran's (1958) process. For the tractability of the complicated equations, we adopted the fitness definition provided by Cooney et al. (2016) that fitness is expressed as the exponential of the expected payoff.

Suppose that the values of R, T, S, P (Figure 1) are affected by the latent variable χ which is analogous to toxicity in the simulation and empirical experiments (R: Reward, T: Temptation, S: Sucker, P: Punishment). Our model postulates that higher toxicity makes the cost of defection, especially mutual defection, more severe. This is expressed as $\frac{dP}{d\chi} < \frac{dS}{d\chi} = \frac{dT}{d\chi} < \frac{dR}{d\chi} < 0$ determined by the number of acting cooperators (the individual who cooperates as a consequence of the correct or incorrect recognition) in both actor and the recipient (0 for P, 1 for S and T, or 2 for R, refer to Figure 1B). Our analysis results indicate that the relative fitness of sharers is positively correlated to toxicity (Proposition 2). In other words, defectors are relatively advantageous if toxicity is low. A detailed explanation of the whole propositions, proofs, corollaries, and definitions is provided in Supplementary Material.

We calculated the fitness of each sharer against the frequency of the sharers in the population without a like-with-like assortment. Through this process, we expected that the relative fitness of sharers is frequency dependent (Proposition 3.1, 3.2). That is, the existence of more sharers increases the fitness of each sharer. This proposition explains why the fitness of flocculative yeast cells from our experiments is in proportional to their frequency in the presence of chemical stress.

To analyze the effect of the accuracy of distinction (or recognition error) on the fitness of the sharers, we adopted definitions of favor in *strong* and *weak sense* from Cooney et al. (2016). According to these definitions, the accuracy of distinction favors the green-beard gene in a *strong sense* if the increasing accuracy

of distinction escalates the expected frequency of the gene in the subsequent step regardless of the current gene frequency (Definition 1). Similarly, the accuracy of distinction favors the green-beard gene in a *weak sense* if increasing the accuracy of distinction escalates the fixation probability of the gene (Definition 2). For the case of yeasts under external stress, we assumed that $R > T = S > P$ as R is the payoff of the strong adhesion, S and T are that of the weak adhesion, and P is that of the no adhesion (refer to the biological interpretation in Supplementary Material). This condition was defined as "flocculating-yeast game." Our analyses concerning recognition error show that the accuracy of distinction to sharers (β_S) favors green-beard genes in both *strong and weak sense* in the flocculating-yeast game ($R > T = S > P$) as well as in the prisoner's dilemma game ($T > R > P > S$) and the hawk–dove game ($T > R > S > P$) if conditions of $S + T - 2P > 0$ and $R + P > S + T$ are satisfied (Propositions 4, 5). Interestingly, if the sharer frequency is low, decreasing accuracy of distinction to the defectors (β_D) confers greater fitness (Proposition 6.2). Although we could not experimentally modify the accuracy of distinction, Proposition 6.2 explains the conditions when permissive heterophilic adhesion rather than homophilic adhesion could be more advantageous.

Experiment

Our mathematical predictions address that the fitness of the green-beard gene is frequency dependent and affected by external stress. To test our predictions, we used yeast *FLO1* that enables yeast cells to aggregate in the harsh condition which is proven to satisfy the condition of the green-beard gene (Smukalla et al., 2008). The induction of *FLO1* and the secretion of flocculin is analogous to cooperation in the mathematical model, while not secreting flocculin is analogous to defection.

Suppose that the cells are exposed to chemical stress. It is advantageous for cells to be situated in the core of the flocs. To be a part of the floc, cells should either secrete the flocculin or be attached by other flocculin-secreting cells. The results of Smukalla et al. (2008) show that cells that do not secrete flocculin also can be included in the floc due to the molecular traits of the flocculin that adhere to cells without flocculin (Soares, 2011). This is against the traits of the green-beard gene, which postulates exclusive cooperation among the sharers and thus can be regarded as a recognition error.

The values of the R, T, S, P for yeast cells can be determined by external stress. Secretion of flocculin entails costs but increases

the probability to be included in the floc, which is crucial for survival when the external stress is high. In this condition, the payoff of the mutual cooperation (R) would be higher than mutual defection (P) as it is likely that mutually cooperating cells are included in the floc, while mutually defecting cells that are likely to be planktonic become susceptible to external stress. In contrast, mutual defection is more advantageous if there is no external stress as inclusion in the floc has no advantage while secretion of flocculin entails cost. In addition, the frequency of the conspecifics also affects the relative fitness of each cell. As certain amounts of *FLO1*^{+} cells are required to form the flocs, dispersed *FLO1*^{+} cells cannot form a floc that would protect them from external stress. It can be interpreted as a decreased probability to encounter cooperators in the game theory (in the absence of a like-with-like assortment). To empirically verify the validity of the mathematical theory, we prepared different proportions of yeast cells with or without *FLO1* and applied different chemical stress (Figure 2).

We measured the relative fitness of *FLO1*^{+} cells under different initial *FLO1*^{+} cell proportions and stress. We used the generalized linear mixed-effects model (GLMM) to evaluate the significance of the initial proportion and stress intensity. The stress index multiplied by the initial proportion (the interaction term in the GLMM) was a significantly positive factor for the fitness (ANOVA for the mixed-effects model, $F_{1,49} = 38.729$, $P < 10^{-6}$). Therefore, initial proportion and stress were positively correlated with the relative fitness of the *FLO1*^{+} cells. In the absence of stress, *FLO1*^{+} cells exhibited less fitness, corresponding to the previous observation (Smukalla et al., 2008) and validating the inclusion of the baseline cooperator cost (Δ) in the model. The relative fitness values and statistical analyses based on different assumptions about mass and CFUs are presented in Supplementary Material.

Simulation

We prepared a square lattice of size 100 × 100 on which agents—cooperators or defectors—play the iterated game with their edge-sharing neighbors (see Figure 4A). Cooperators cooperate with cooperators while defects to the defectors as formulated by the traits of the green-beard gene. Cooperators defect to the cooperator or cooperate with the defector if a recognition error occurs. Defectors invariably defect regardless of the recognition error. The payoff of the simulation is determined by basic fitness (w_0), external stress (χ), and the cost to be included in the floc. Specifically, the

probability to be included in the floc for mutual cooperation (adhesion) is $\phi_2(p) = [1 + \exp(-10(p - 0.5))]^{-1}$ (logistic relation, which is used to describe chemical reactions; Cramer, 2003; Reed & Joseph Berkson, 2002) where p is the frequency of the cooperators. The probability to be included in the floc for the opposite actions (one cooperation and one defection) is $\phi_1(p) = 0.85\,\phi_2(p)$. Therefore, $\phi_2(p) \geq \phi_1(p)$, and they are the increasing functions of p. The maximal probability of inclusion into the floc was set to be 0.7. Thus, defection increases the probability not to be included in the floc, making the cell vulnerable to external stress. The cost to be included in the floc (isolation from nutritious media and different gene expressions; Smukalla et al., 2008) is a constant k. Included in the floc it may be, the periphery of the floc is mostly inhabited by defective cells as shown by the previous empirical observation (Smukalla et al., 2008). This is reflected in the condition $\alpha_S > \alpha_P > 0$, the coefficients for the external stress (χ) that describes the damage under chemical stress. Hence, the payoff values are assumed to be

$$R = w_0 - k\phi_2(p), \tag{5}$$

$$S = T = w_0 - \alpha_P\chi - k\phi_1(p), \tag{6}$$

$$P = w_0 - \alpha_S\chi. \tag{7}$$

Based on the payoff values, agents on lattice play games with their neighbors and accumulate the payoff, which later becomes fitness. All cooperators have fitness costs of becoming cooperative (the secretion of flocculin, corresponding to Δ of Equation 3). The fitness of each agent determines the probability to replicate its copy, changing the proportion of cooperators or defectors in the subsequent generation. This proportion after several generations of iterated games was measured by changing the initial proportion and stress intensity. Three sets of simulations were performed: (a) Changing accuracy rates ($\beta_S = \beta_D$) while setting the cooperation cost fixed ($\Delta = 0.6$); (b) changing cooperation costs (Δ) while setting the accuracy rates fixed ($\beta_S = \beta_D = 0.9$); and (c) changing the accuracy rate to defectors (β_D) while setting other conditions fixed ($\beta_S = 0.9$, $\Delta = 0.6$). The results of each simulation set are presented in Supplementary Animations S1–S3, respectively.

The results of our numerical simulation based on the aforementioned assumptions show that, as expected by mathematical analysis, the relative fitness of the cooperator monotonically increases against toxicity across all conditions (Supplementary Animations S1–S3). For a given accuracy rate, the relative fitness against the initial proportion monotonically increases if the toxicity exceeds a certain threshold. For example, the fitness of a
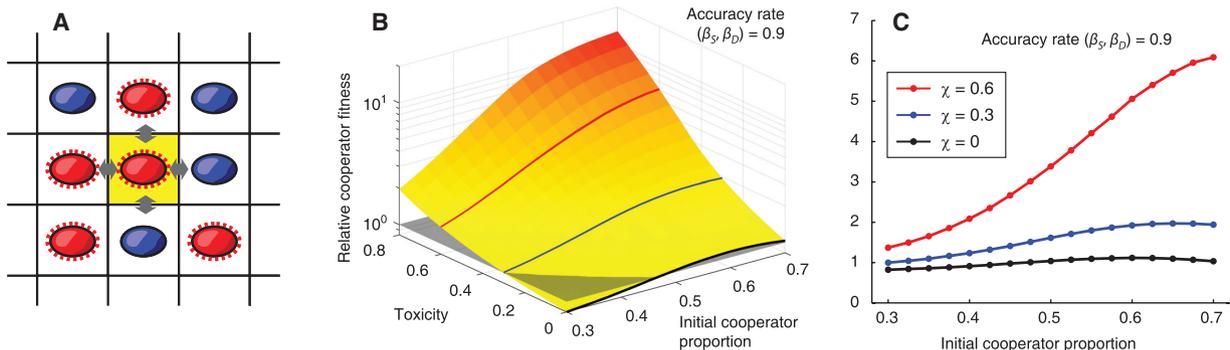

Figure 4. The design of the simulation and the results. (A) An individual in each grid sequentially plays the two-person game with other individuals in the neighborhood (left, right, top, bottom). (B) The fitness surface of the cooperators against toxicity and initial cooperator proportion when both accuracy rates are 0.9. The cost of cooperation (Δ) is 0.6. The transparent gray flat surface illustrates the relative fitness of 1. The black, blue, and red lines along the surface indicate the fitness against the initial proportion given that toxicity (χ) values are 0, 0.3, and 0.6, respectively. These lines are shown in (C). (C) The fitness of the cooperator given that toxicity values are 0 (black), 0.3 (blue), and 0.6 (red).

cooperator is a monotonically increasing function of initial proportion in high stress (χ = 0.6) given that both accuracy rates (β_S, β_D) are 0.9 (red lines in Figure 4B and C). The relative fitness of the cooperators against the initial proportion of the cooperators is nearly flat yet slightly concave in the absence of stress (χ = 0) (see Figure 4C). The general schematics of the relative fitness measured by empirical experiments and simulation in the absence of stress (EtOH 0%, χ = 0), negligible stress (EtOH 10%, χ = 0.3), and high stress (EtOH 20%, χ = 0.6) are similar (Figures 4C and 5D) in that initial frequency and severe stress elevated the cooperator fitness.

Increasing cooperation cost (Δ) lowers the general elevation of the fitness surface, while it does not change the general schematics of the surface (Supplementary Animation S2). The accuracy rate to the defectors (β_D) with the fixed accuracy rate to the cooperators has differential effects depending on the initial cooperator proportion (Supplementary Animation S3). Low β_D is advantageous for the cooperators if the cooperator frequency is low and the stress is severe (Figure 6C).

Discussion

We explored the factors that affect the evolutionary stability of green-beard genes including the recognition error. We built mathematical formulations whose expectations were tested by simulations and empirical experiments. First of all, higher values of R and S make the green-beard genes stable, while the same holds true for lower values of T and P (indicated by Proposition 1). In the game-theoretic aspect, it can be interpreted as the relatively low cost or high reward of cooperation bolsters the stability of the green-beard gene. Severe chemical stress to yeasts confers a higher relative payoff to the adhesion (cooperation), and this would have resulted in higher fitness of $FLO1^+$ cells under EtOH 20% condition (Figure 5C). This case is described in Proposition 2.

Second, more sharers boost the probability of sharers to spread if the accuracy of distinction and cost of mutual defection is high. According to our analysis, the relative fitness of the green-beard gene is an increasing function of the sharers (Proposition 3.1, 3.2). More specifically, there is a specific number of sharers that determine whether $\pi_i^S - \pi_i^D$ is negative or positive. Consequently, the fixation of the green-beard gene or defector gene is a positive feedback process. For the first time to our knowledge, we experimentally examined the frequency-dependent fitness of flocculating yeasts under different stress conditions.

The general framework of our model is similar to previous theoretical models on the green-beard effects in that the probability to interact with carriers and noncarriers of the green-beard genes with the costs is considered (see Gardner & West 2010 and Biernaskie et al. 2011 for details). As an expansion of previous frameworks that interactions and the corresponding payoff among cooperators or defectors are solidly determined by their genotypes and frequency, our model incorporated stochastic recognition error that diversified the interactions (e.g., a cooperator may defect another cooperator). In addition,
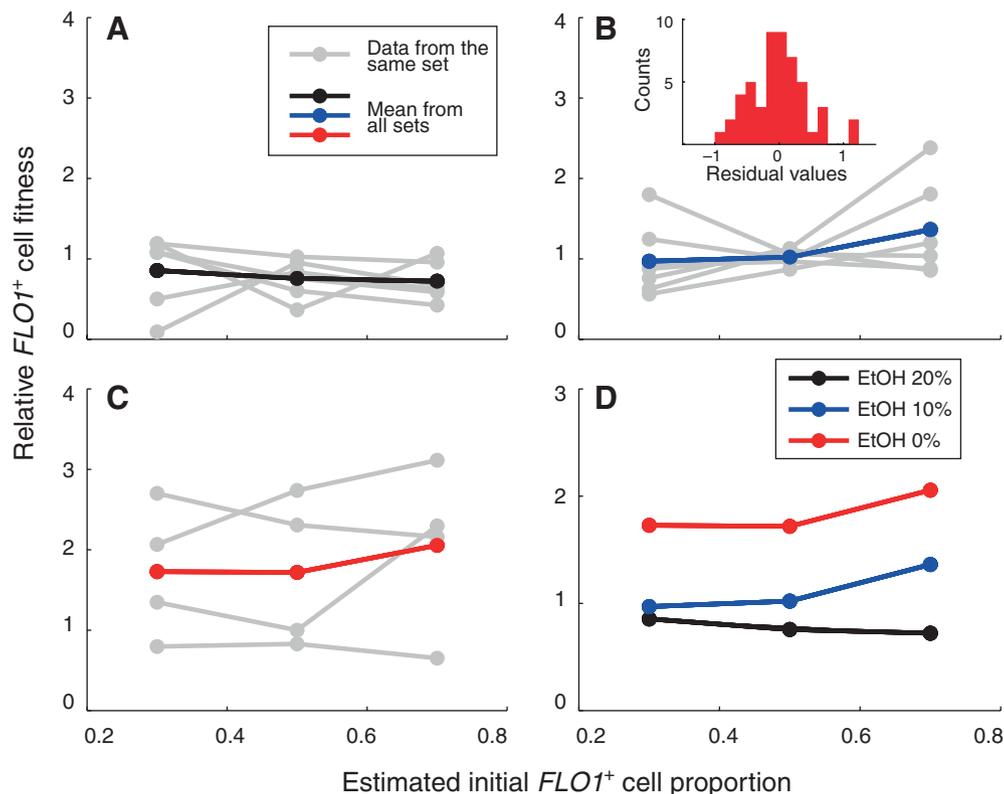

Figure 5. The relative fitness of $FLO1^+$ (flocculating) cells compared with $flo1^-$ (nonflocculating) cells. (A–C) The estimates of $FLO1$ relative fitness with different initial proportions and stress intensity. The data from the same set were linked with gray lines. The same set here refers to the cells from the same culture, which are expected to share similar viability. Three conditions of chemical stress were tested: 0% (A), 10% (B), and 20% EtOH (C). The inset of (B) represents the histogram of residuals obtained from the generalized linear mixed-effects model used in the statistical analysis. The contour of the histogram is similar to the normal distribution. (D) The average relative fitness in different stress conditions. Three lines are average (colored) lines from (A–C).
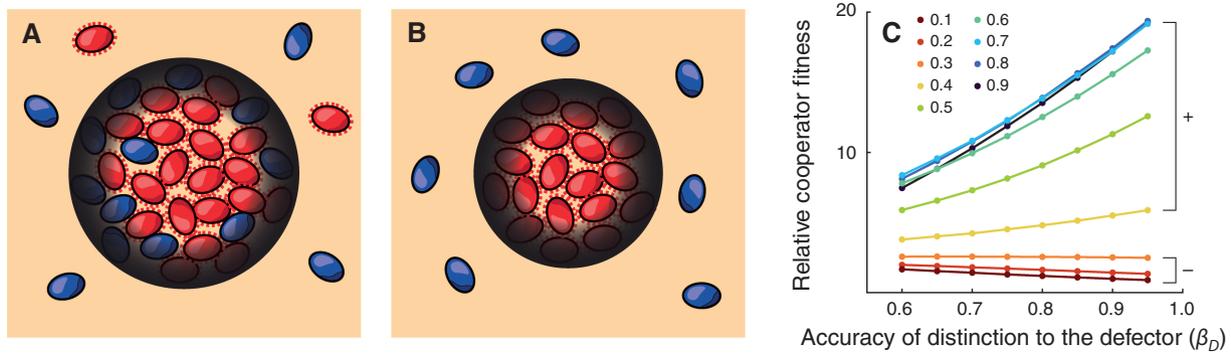
Figure 6. Cooperation with defectors by adopting recognition error to the defectors could be evolutionarily advantageous if the cooperator frequency is low and the cost of mutual defection is severe. (A) If the flocculating cells are scarce, forming adhesion with nonflocculative cells (cooperation) can induce larger flocs where most nonflocculative cells are located at the periphery of the floc. Forming adhesion with nonflocculative cells may preclude the inclusion of several flocculative cells into the floc. However, as the peripheral region of the floc is damaged from external stress, the total survival of the flocculative cells could be higher if they bind with the nonflocculative cells. The susceptible part in the floc is illustrated with the dark transparent circular ring. (B) If the flocculating cells form a floc purely with its infrequent conspecifics, the floc size would be small, and most part of the floc will be susceptible to external stress. This strategy is less advantageous than cooperating with nonflocculative cells. (C) Using our simulation model, we measured the relative fitness of the cooperators with different cooperator proportions and the accuracy of distinction to the defectors (β_D). The colored dots in the upper left region shows the cooperator proportion from 0.1 to 0.9. As expected by Proposition 6.2, low β_D is advantageous for the cooperator fitness when the cooperator frequency is low. If the cooperator proportion is 0.1, 0.2, or 0.3, then the slopes of the lines are negative, indicating that increasing β_D is disadvantageous (marked with a "–" sign). For other proportion values, the slope is positive (marked with a "+" sign). Toxin intensity (χ) and accuracy of distinction to the cooperators (β_C) were both fixed as 0.9. The general fitness surface is presented in Supplementary Animation S3.

payoff values in our simulation models are determined by stress intensity and total cooperator density, which are specialized for the flocculation of yeasts. Our results of a positive correlation between the fitness of the green-beard gene and its frequency are in the same line with the conclusions of the previous models (Biernaskie et al., 2011; Gardner & West, 2010). Empirical findings support these analysis results. Before the stalk formation gene (*csA*) of social amoeba was recognized as a green-beard gene (Queller et al., 2003), Buss (1982) discovered that the frequency of the stalk-forming cooperators affects the fitness of the cooperators.

Like-with-like (positive) assortment, indicating frequent interactions among the cooperators, has been proposed as the mechanism for the evolution of cooperation (Cooney et al., 2016; Pepper & Smuts, 2002). Frequency-dependent fitness of our model supports this claim as positive assortment increases the local frequency of cooperators in the region (Madgwick et al., 2019). Although strict initial takeoff condition was pointed out as the implausibility of the green-beard gene in the real world (Madgwick et al., 2019), we propose that the green-beard gene may thrive in a large population if a population grows with the reproduction or constant immigration. Once a small population is dominated by the green-beard gene due to selection or genetic drift (Kimura, 1968), then steady growth of the population afterward ensures the frequency of green-beard genes to be sufficiently high to spread. Such a population dominated by the green-beard gene also can be formed by the founder effect, at least at the initial stage of the primary succession (Brislawn et al., 2019). For example, a small number of yeast spores may land on a fertile yet unoccupied surface to form large colonies.

In a rough sense, the conditions for Proposition 3.1 (theoretical basis of frequency-dependent fitness) indicate $R \gg P$ and $S \gg P$. Hence, one may come up with hawk–dove games ($T > R > S > P$) satisfying the conditions provided in Proposition 3.1. We also defined the "flocculating-yeast game" ($R > S = T > P$), which can satisfy the conditions of Proposition 3.1 as well. (See Supplementary Material for more details.) These conditions are

in the same line with Proposition 2 (theoretical basis of stress-dependent fitness) embracing the toxicity effect (χ). Given that $\frac{dR}{d\chi} > \frac{dS}{d\chi} = \frac{dT}{d\chi} > \frac{dP}{d\chi}$ holds with some range of initial values (R, T, S, P when $\chi = 0$), increasing toxicity can generate hawk–dove game or the flocculating-yeast game. High chemical stress corresponds to a higher cost of mutual defection as failure to cooperate (being excluded from the floc) results in the death of the cell. Increasing relative fitness of *FLO1⁺* cells under EtOH 20% stress corroborates this claim (Figure 5D). The fitness of cooperators in the numerical simulation also increased under high toxicity (Figure 4C).

The accuracy of distinction among the sharers (β_S) favors green-beard genes in the *strong* and *weak sense* given some constraints that may satisfy the condition of the flocculating-yeast game, prisoner's dilemma game, or hawk–dove game. (Proposition 4, 5) This can be interpreted that the ability to distinguish whether the opponent carries the green-beard gene is crucial for its spread. The relative fitness of cooperators in the numerical simulation (flocculating-yeast game) increased with increasing accuracy of distinction given that the accuracy of distinction to sharers (β_S) and defectors (β_D) are identical (Supplementary Animation S1). Note that though the accuracy of distinction may not be perfect, the green-beard gene could be evolutionarily stable.

It might be evolutionarily advantageous for yeast cells to adopt the recognition error to defectors. It is not mechanistically impossible for yeast cells to adopt more reliable means of aggregation. For example, *FLO11*, one of the flocculin-encoding genes along with *FLO1*, has the trait of homophilic adhesion (Belpaire et al., 2022; Brückner et al., 2020). Hence, the adoption of *FLO11* rather than *FLO1* as the primary adhesion molecule would enhance the accuracy of the green-beard recognition. Proposition 6.2 suggests that reducing the accuracy of distinction to the defectors (β_D) is advantageous for the green beards of the low frequency in flocculating-yeast games. If conspecific green beards are infrequent, it would be a better option for green beards to cooperate (adhere) with defectors although this gives benefit to the defectors.

We speculate that the adoption of the recognition error of yeasts has two advantages compared to the strict green-beard

recognition ($\beta_D = 1$) in the empirical sense. First, if the purpose of the aggregation is endurance from external stress, there are economies of scale. As external stress infiltrates the periphery of the floc, smaller flocs are not comprehensively protected from the stress (Smukalla et al., 2008). Suppose that the stress infiltrates the part of the floc whose distance to the floc surface is less than r. For a spherical floc of the radius R ($>r$), the susceptible volume is $\frac{4}{3}\pi(R^3 - (R - r)^3)$. Dividing this susceptible volume by the total volume ($\frac{4}{3}\pi R^3$) shows that the proportion of the susceptible volume decreases as the total volume increases (Figure 6A and B). In other words, the average damage of each cell within a floc is reduced if the floc size is huge. Thus, the expansion of the total floc volume is advantageous for each yeast cell. In a mixed population, low β_D can promote the size of the floc by recruiting other nonflocculating cells without flocculins. The benefit of cooperation with nonclonal organisms was discovered in social amoebas (*D. discoideum*) when size dependence comes into play (Foster et al., 2002).

Suppose that the yeast density is low. Then floc would not be large enough to sustain the stress. In this condition, cooperation with defectors (*flo1⁻* cells) is beneficial as the floc size becomes larger. Without the adhesive interaction to the defectors, all cooperators may die. On the other hand, if the yeast density is high, then cooperators can form large protective flocs without forming adhesions with the defectors. In this case, defection to defectors is advantageous, which will result in a homogeneous population of cooperators in the subsequent generations. As the benefit and cost of cooperation with defectors are context dependent, yeast may have retained permissive adhesion as the "insurance" for the stressful condition combined with low cell density.

In addition, *FLO1⁺* cells are located at the central part of the floc, while most *flo1⁻* cells are located at the periphery of the floc providing a protective shell of the floc (Smukalla et al., 2008). This configuration is also expected from the differential adhesion hypothesis considering adhesive forces among *FLO1⁺* and *flo1⁻* cells (Belpaire et al., 2022; Foty & Steinberg, 2005). One may interpret that *FLO1⁺* cells exploit *flo1⁻* cells as expendable shields, although inclusion in the floc even at the periphery increases the fitness of the false beards. This interpretation should be carefully handled as numerous factors including the proportion of cell types, total cell density, and stress intensity would affect the payoff of such exploitation as the protective shield. For example, the inclusion of the *flo1⁻* cells may induce some *FLO1⁺* to be planktonic, becoming susceptible to external stress.

To measure the effect of the β_D in different cooperator frequencies, we additionally performed simulations with fixed $\beta_C = 0.9$ and stress intensity $\chi = 0.9$ with different β_D and cooperator frequency. The simulation results show that increasing β_D when cooperator frequency is low decreases the cooperator fitness (Figure 6C). This is compatible with the indication of Proposition 6.2. Depending on cooperator frequency and the cost of the mutual defection, becoming similar to an obligate cooperator (ALLC; Imhof et al., 2005) could be beneficial in certain conditions. The surface configuration of Supplementary Animation S3 also exhibits an identical trend.

To empirically verify this hypothesis, further studies may compare the fitness of the yeast strains, which use *FLO1* or *FLO11* as the primary flocculin-encoding genes. (The tandem repeats of *FLO1* should be controlled to exhibit similar homotypic adhesion to that of *FLO11* as longer tandem repeats result in stronger adhesion; Verstrepen et al., 2005.) *FLO1⁺* cells can recruit other *flo1⁻* cells as a protective perimeter, while some *FLO1⁺* cells could remain planktonic. In contrast, (wild or genetically modified) *FLO11⁺* cells may form highly homogeneous floc at cost of the protective perimeter composed of different strains. We expect that there is a tradeoff between these two strategies. According to our model frameworks, *FLO1⁺* cells will have higher fitness compared to the *FLO11⁺* cells when the external stress is severe and the proportion of flocculative cells is low. However, the prevalence of *FLO1* in brewing yeasts could be the consequence of artificial selection as permissive adhesion would be suited for removing the yeast cells during brewing. In addition, homotypic *FLO11* rather than *FLO1* might be prevalent in wild yeasts (Zara et al., 2009). The evolutionary tradeoff of heterotypic permissiveness and homotypic strictness requires further investigation.

We were not able to measure the proportion of the *FLO1⁺* cells in the flocs and suspension separately due to technical difficulty. Such measurement would enable estimation of how often recognition error occurs at specific defector frequency, which could be the topic of subsequent studies. Further studies should focus on the measurement of β_S or β_D, and manipulation of those values to test the validity of the theory presented in this study. Weaker or stronger expression of *FLO1* or other flocculin-encoding genes, such as *FLO5*, which weakly correlate with flocculation of cells (Smukalla et al., 2008) or application of chemicals that selectively facilitate or inhibit the flocculation, could be the method to manipulate the accuracy of distinction. Change of the rotor amplitudes could be one way to change the accuracy of distinction, as greater shear force detaches the weakly attached cells, resulting in smaller flocs composed mostly of *FLO1⁺* cells (Belpaire et al., 2022).

In conclusion, we utilized the game theory to build the analytical model for green-beard effects considering the effect of the recognition error. Well beyond theories, we linked our analysis to empirical experiments using yeasts and performed a simulation of the iterated game. We also suggested a novel viewpoint that recognition error to the defectors could be evolutionarily beneficial in certain contexts. To our knowledge, no model thus far embraced the effect of recognition error with empirical confirmation. Our findings will provide insights into both theoreticians and practitioners—Theoreticians should consider the error effect when estimating the stability of the green-beard gene to build reliable and realistic models, and practitioners should measure the error rate of recognition to solidify the theories of the green-beard effect.

Supplementary material

Data availability

Author contributions

Conceptualization: J.C. Mathematical analysis and simulation: J.C., J.P. Experiments: J.C., S.L., H.K. Initial writing: J.C., S.L. Revision and review: J.C., S.L., H.K., J.P.

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