**Yeast**
*Yeast* 2008; **25**: 41–46.
Published online 8 October 2007 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1552

**Research Article**

# The spectrum of Trp⁻ mutants isolated as 5-fluoroanthranilate-resistant clones in *Saccharomyces bayanus, S. mikatae* and *S. paradoxus*

Elizabeth W. Jones*, Peter B. Berget, James M. Burnette III, Candice Anderson, Denise Asafu-Adjei, Seda Avetisian, Fatmata Barrie, Ruby Chen, Bur Chu, Samantha Conroy, Sean Conroy, Allyson Dill, Will Eimer, Diane Garrity, Alexander Greenwood, Tamara Hamilton, Simon Hucko, Carmen Jackson, Kristen Livesey, Tiffany Monaco, Christina Onorato, Mai Otsuka, Satyan Pai, George Schaeffer, Sharon Shung, Samantha Spath, Jonathan Stahlman, Blake Sweeney, Elizabeth Wiltrout, Daniel Yurovsky and Andrea Zonneveld
*Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA*

**Correspondence to: Elizabeth W. Jones, Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213, USA.*
*E-mail: ej09@andrew.cmu.edu*

Received: 19 July 2007
Accepted: 7 August 2007

## Abstract

**5-Fluoroanthranilic acid (FAA)-resistant mutants were selected in homothallic diploids of three *Saccharomyces* species, taking care to isolate mutants of independent origin. Mutations were assigned to complementation groups by interspecific complementation with *S. cerevisiae* tester strains. In all three species, *trp3, trp4* and *trp5* mutants were recovered. *trp1* mutants were also recovered if the selection was imposed on a haploid strain. Thus, FAA selection may be more generally applicable than was previously described. Copyright © 2007 John Wiley & Sons, Ltd.**

**Keywords:** **5-fluoroanthranilate; *Saccharomyces* species; *trp* mutants; mutant selection**

## Introduction

The ability to counterselect gene function greatly expands the capability for genetic analysis and manipulation of cells. A few methods for counterselection of genes in the yeast *S. cerevisiae* have been developed, but the use of 5-fluoro-orotic acid (5FOA) to counterselect the *URA3* gene dominates (Boeke *et al*., 1984). A similar counterselection of genes encoding tryptophan biosynthetic enzymes that employs 5FAA has been reported (Toyn *et al*., 2000). An unusual feature of the mutant spectrum reported for the latter experiment was that all 144 newly isolated mutants were *trp5* mutants, although evidence was provided for three different kinds of *trp5* alleles. Assuming that FAA is toxic via conversion to 5-fluorotryptophan, one would expect to recover mutations in all genes whose products are required for conversion of anthranilate to tryptophan: *TRP1, TRP3, TRP4* and *TRP5* (Figure 1). As no information was provided about the method of recovery of the mutants in Toyn *et al*. (2000), whether from liquid culture or from independent colonies, e.g. the significance of this narrow mutant spectrum was unclear.

As part of an undergraduate summer research programme, we elected to develop the molecular genetics of several homothallic *Saccharomyces* species whose genomes had been sequenced: *S. bayanus, S. castellii, S. mikatae* and *S. paradoxus* (Cliften *et al*., 2001; Kellis *et al*., 2003). All save *S. castellii* are members of the *Saccharomyces sensu strictu* group (Naumov *et al*., 2000; Petersen *et al*., 1999). As part of that effort we selected for FAA-resistant colonies, screened for Trp⁻ mutants, and then assigned the mutations to complementation groups by interspecific complementation tests to *S. cerevisiae* tester strains. *trp5*

Copyright © 2007 John Wiley & Sons, Ltd.

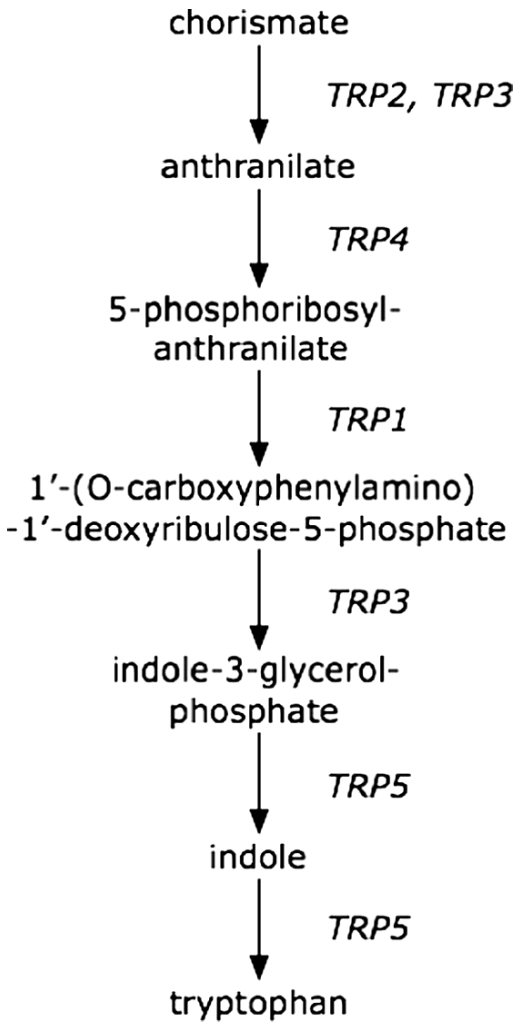


**Figure 1.** Tryptophan biosynthesis. Gene names are placed next to the steps for which their products are required

mutants constituted the most abundant class. However, *trp3* and *trp4* mutants were also fairly common and recovered from all three species. No *trp1* mutants were recovered. *trp1* mutants, along with mutants for the other three *TRP* genes, were found among FAA-resistant mutants of a *MAT***a** haploid *S. bayanus*, although the *trp1* mutants were the least frequent class. Thus, FAA selection is more broadly applicable than had previously been established.

## Materials and methods

### Strains

Samples of the *Saccharomyces* species were obtained from Mark Johnston (Washington University, St. Louis, USA). The species we chose to work with were *S. bayanus, S. castellii, S. mikatae* and *S. paradoxus*. *S. castellii* is evolutionarily the most distant. Table 1 includes the designations for the strains used. All four species were homothallic.

### Media

YPD and synthetic media (C, C-trp, and MV) were prepared for yeast cultures as previously described (Jones *et al*., 1982; Zubenko *et al*., 1982). FAA (Toyn *et al*., 2000) and 5-fluoro-orotic acid (5FOA) (Boeke *et al*., 1984) media were prepared as described. MV + trp, MV + anth, and MV + ind contain 40 mg/l tryptophan, 50 mg/l anthranilate and 50 mg/l indole, respectively, added to MV

**Table 1.** Strain list

| Laboratory designation | Strain | Other designations |
|---|---|---|
| BJ10501 | FM361 *Saccharomyces bayanus** | NRRL Y-11845, CBS 7001, MCYC623, 623-6C |
| BJ10503 | FM360 *Saccharomyces paradoxus** | N17 |
| BJ10505 | FM356 *Saccharomyces mikatae** | IFO 1815 |
| BJ10507 | FM476 *Saccharomyces castellii** | NRRL Y-12630, ATCC 76901, CBS 4309, IFO 1992 |
| BJ10635 | *MAT***a** *Saccharomyces bayanus*** | |
| BJ528 | *MATα trp3 leu1 ura3 lys7 his5 met1 pet17* | |
| BJ2743 | *MATα trp4 leu2-3 pet2 arg4 aro1C rna3* | |
| BJ2749 | *MATα trp2 his6 ura1 ino1 leu2-3 MAL1 ts11* | |
| BJ3038 | *MATα trp5 lys2* | |
| BJ3039 | *MAT***a** *trp5 lys2* | |
| BJ8918 | *MAT***a** *trp1 his3-Δ200 ura3-52 leu2* | |
| BJ8920 | *MATα trp1 his3-Δ200 ura3-52 leu2* | |

* From M. Johnston, Department of Genetics, Washington University, St. Louis, MO, USA.
** The haploid isolate was recovered from a streak of FM361.

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/yea

medium. PSP contains 10 g/l potassium acetate, 8 g/l nutrient broth and 10 g/l yeast extract. Sigma agar was added at 14 g/l for solid media.

### Genetic methods

To isolate Trp$^-$ mutants from the homothallic diploids, strains were streaked for single colonies on YPD. Single colonies were picked to a YPD master at 16 or 32 colonies/master plate. After 2 days at 30 °C (instructor) or 32 °C (students), the YPD master plates were replica-plated to two PSP plates for sporulation, two 5FOA- and two FAA-containing plates. The PSP plates were incubated at 30 °C or 32 °C. After 4 days incubation, the PSP plates were replica-plated to two FAA- and two FOA-containing plates. One FAA- and one FOA-containing plate pre- and post-sporulation were irradiated for 15 sec, 35 cm from a 30 W UV light bulb. All selective plates were incubated for 3–4 days at 30 °C or 32 °C. Papillae arising in the replica-plated patches were picked from FAA plates to YPD master plates. After 2 days growth at 30 °C or 32 °C, they were replica-plated to C, C — Trp and YPD media and incubated for 2 days at 30 °C or 32 °C. Trp$^-$ mutants (or sectors of streaks, since cells from the background were often transferred) were picked to YPD master plates. After 2 days of growth at 30 °C or 32 °C, they were replica-plated to MV, MV + anth, MV + ind, MV + trp media. The plates were scored for growth at 1 or 2 days. Growth of papillae was robust for *S. mikatae* and *S. bayanus*; background growth was more of a problem for *S. paradoxus*. For mutagenesis of haploids, individual colonies were picked to YPD plates and, after 2 days growth at 32 °C, the YPD masters were replica-plated to two FAA plates. One plate was irradiated with UV light for 5 min. Papillae were picked and tested for growth as for the diploid mutants.

To carry out interspecific complementation tests on diploid mutants from the homothallic strains, eight Trp$^-$ mutants at a time were streaked the full width (top to bottom) of a YPD plate. After 2 days at 30 °C or 32 °C, the plates were replica-plated to PSP. The interspecific complementation tests were designed to take advantage of meiosis to generate spores of both mating types that could mate with the *S. cerevisiae* testers. However, we did not test whether sporulation was actually occurring in the mutant streaks on PSP. Conceivably, mating cells were generated by mitotic recombination or by heterothallic switching in the tester strains. For the purposes of the complementation tests this does not really matter, so long as they work. After 4 days at 30 °C or 32 °C, the PSP replicas were replica-plated to YPD and immediately cross-stamped with *trp* tester strains using the long edge of sterile tongue depressors. After 2 days incubation at 30 °C or 32 °C, the cross-stamped plates were replica-plated to C — Trp or MV plates. Trp$^+$ growth at the intersection indicated complementation between the *trp*$^-$ mutation in the mutant and that in the tester. The test worked very well for *S. bayanus*, quite well for *S. mikatae* and weakly but still definitively for *S. paradoxus*. No data were obtained for *S. castellii*, since the strain proved to be naturally FAA$^R$ but Trp$^+$. None of the strains gave FOA$^R$ Ura$^-$ colonies.

Strains were sporulated on PSP and dissected in the usual way. Only *S. mikatae* gave abundant tetrads.

## Results and discussion

We chose to start this study with four different species of *Saccharomyces*, viz. *S. bayanus, S. castellii, S. mikatae* and *S. paradoxus. S. paradoxus* is the most closely related to *S. cerevisiae*, followed by *S. mikatae* and then *S. bayanus.* All three are members of the *sensu strictu* group (Naumov *et al*., 2000). *S. castellii* is more distantly related and belongs to the *sensu lato* group (Petersen *et al*., 1999). All four species are homothallic and apparently diploid. Because of this, it seemed necessary to incorporate a sporulation regimen into the mutagenesis protocol to generate haploid mutants, which we expected to subsequently mate with their daughters and be isolated as homozygous diploids. Individual colonies were picked from a YPD streak plate onto YPD. Once grown, the colonies were replica-plated to two plates each of selective media (FAA, FOA) and to PSP to elicit sporulation. The sporulation plates were replica-plated to selective media after 4 days incubation. One member of each pair of selective plates, whether replicated from the YPD master or the PSP replica, was irradiated with UV light to induce mutations.

As expected, few papillae appeared on the FAA plates for the homozygous diploids replica-plated from YPD masters, whether or not the plates were

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/yea

irradiated (although more papillae appeared on the irradiated plates). There was a substantial increase in the number of papillae/streak, of the order of 5- to 10-fold, after cells had been incubated on the PSP sporulation medium. Again the irradiated plates had substantially more papillae. Thus, incubation under conditions that elicit meiosis and sporulation and mutagenesis resulted in a much higher frequency of mutation to $FAA^R$. Independent papillae were picked and tested for a tryptophan requirement and for the ability to use indole to satisfy the tryptophan requirement.

The $Trp^-$ mutants were tested for complementation and assigned to complementation groups, using *S. cerevisiae* testers. Representative complementation tests are presented in Figure 2. In the left half of the figure are presented results for the diploid mutants obtained from the homothallic strains; replicas were sporulated 4d on PSP before being replica-plated for cross-stamping and complementation testing. Columns 1–3 in this plate show the results for diploid *trp3, trp4* and *trp5* mutants of *S. paradoxus*; columns 4–6 show results for diploid *trp3, trp4* and *trp5* mutants of *S. mikatae*; column 7 shows results for a diploid *trp4* mutant of *S. bayanus*; and column 8 for a haploid *trp5* mutant of *S. bayanus*. The diploid mutants all show complementation with both **a** and $\alpha$ *trp1* testers, a result expected, since spores of both mating types should be present in the sporulated patches. Similarly, all save the *trp5* mutants complement both **a** and $\alpha$ *trp5* testers. By contrast, the *MAT***a** haploid *trp5* mutant of *S. bayanus* complements the $\alpha$ but not the **a** *trp1* tester and fails to complement the *trp5* testers of either mating type. Complementation tests for *trp1, trp3, trp4* and *trp5* mutants isolated in a *MAT***a** haploid strain of *S. bayanus* are shown in the right half of Figure 2. As expected, the mutants show complementation only with *MAT*$\alpha$ testers. Test results for two each of *trp1, trp3, trp4* and *trp5* mutants are shown in the right hand plate. No sporulation regimen was necessary for these tests of the haploid mutants.

The pooled results for the three diploid homothallic species are summarized in Table 2. In part A are presented the results from 16 students in the 2004 programme (mutants from the vegetative and sporulation regimens are combined); the results for the instructor's (E.J.) sporulation set are presented in part B and for the instructor's vegetative set in part C of Table 2.

There are several obvious features in these data. *trp5* mutants are by far the most common mutants recovered in each of the three species, whether isolated by students or by an experienced yeast geneticist. Nonetheless, it is equally clear that *trp3*

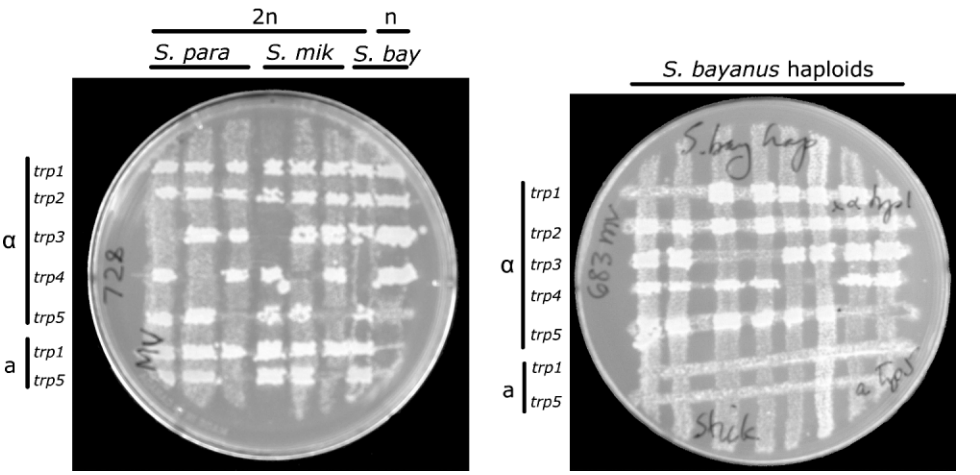


**Figure 2.** Interspecific complementation tests. Clones to be tested were streaked the full width of a YPD master from top to bottom. In the plate to the left, all save the rightmost columnar streak are homothallic diploids; the rightmost streak is a *MAT***a** *S. bayanus* haploid. All columnar streaks in the plate to the right are *MAT***a** *S. bayanus* haploids. After 2d at 32 °C, the plate on the left was replica plated to PSP. After 4d at 32 °C, the PSP plate was replica plated to YPD and immediately cross-stamped with *MAT*$\alpha$ *trp1-trp5* testers and *MAT***a** *trp1* and *trp5* testers. After 2d incubation at 32 °C, the plates were replica plated to MV. Results were recorded at 2d. The plate on the right was treated similarly except the PSP replica was not made; porulation was not induced

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/yea

**Table 2.** Spectrum of Trp⁻ mutants selected by FAA resistance in three *Saccharomyces* species

| | | | | | | Among *trp5* mutants | |
|---|---|---|---|---|---|---|---|
| **Group** | **Total** | ***trp1*** | ***trp3*** | ***trp4*** | ***trp5*** | ***Ind*$^U$** | ***Ind*$^S$** |
| A. Students | | | | | | | |
| *S. bayanus* | 172 | 0 | 30 | 39 | 103 | | |
| *S. mikatae* | 150 | 0 | 36 | 1 | 113 | | |
| *S. paradoxus* | 105 | 0 | 3 | 14 | 88 | | |
| B. Instructor (sporulation regimen) | | | | | | | |
| *S. bayanus* | 75 | 0 | 4 | 15 | 55 | 4 | 1 |
| *S. mikatae* | 71 | 0 | 11 | 6 | 54 | 6 | 6 |
| *S. paradoxus* | 69 | 0 | 2 | 22 | 45 | 3 | 0 |
| C. Instructor (vegetative regimen) | | | | | | | |
| *S. bayanus* | 7 | 0 | 0 | 2 | 5 | | |
| *S. mikatae* | 0 | 0 | 0 | 0 | 0 | | |
| *S. paradoxus* | 30 | 0 | 0 | 3 | 27 | | |
| D. Vegetative haploids | | | | | | | |
| *S. bayanus* | 402 | 9 | 44 | 104 | 245 | | |

and *trp4* mutants are recovered by FAA selection with fair frequency in all three species. It came as a surprise, however, that *trp1* mutants were not recovered. Toyn *et al*. (2000) were able to select *trp1* segregants that had lost a *TRP1*-bearing plasmid by selection on FAA. Thus, it seemed unlikely that FAA is toxic to *trp1* mutants in these three species. We were able to test this for *S. bayanus*. A *S. bayanus trp1* mutant was transformed to Trp$^+$ using pRS314 (a YCp vector) and pRS424 (a YEp vector). After streak purification, several transformants from each vector type were inoculated into YPD broth. After overnight growth, the cultures were streaked on FAA and on YPD to test for loss of the plasmid and selection for FAA$^R$. In each case FAA$^R$ Trp$^-$ segregants were recovered from the transformants. Thus, the low frequency of *trp1* mutants seems unlikely to have a metabolic explanation or result from toxicity.

All three *trp5* allelic forms were recovered, albeit not in all three species (Table 2, part B). All three species yielded *trp5* mutants able to utilize indole for growth (Ind$^U$). Both *S. bayanus* and *S. mikatae* yielded *trp5* mutants that secrete indole (Ind$^S$); only mutants in parts B and C were classified as Ind$^S$. The remainder of the mutants neither utilized nor secreted indole. The number of indole utilizers should be accurate, since these were scored by growth on indole plates. The number of indole secretors is a minimal estimate; they were detected as secretors only if they were sufficiently close to a *trp3, trp4*, or *trp5* (Ind$^U$) strain to cross-feed that mutant.

All of the FAA$^R$ papillae from colonies that had not undergone the sporulation regimen were picked and tested (Table 2, part C). 96 or 128 papillae were picked from the post-sporulation selections (Table 2, part B). Clearly, the yield of mutants was greater in the post-sporulation populations.

We were puzzled by the absence of *trp1* mutants among the Trp$^-$ mutants. As *TRP1* differs from the other loci in mapping very close to its centromere, we elected to test whether we would recover *trp1* mutants if the FAA selection was carried out in a haploid strain. Clearly we recovered *trp1* mutants (Table 2, part D), although they were the least frequent class among the four genes identified. *trp5* mutants were again the most frequent. Although some features of the mutational spectrum may be related to gene size (*TRP1*, 673 bp; *TRP3*, 1454 bp; *TRP4*, 1142 bp; *TRP5*, 2123 bp), this cannot explain the absence of *trp1* mutants recovered in the diploids.

There is no completely satisfactory explanation for the ability to recover *trp1* mutants from haploid but not diploid strains of these homothallic yeasts. However, *TRP1* is distinguished from *TRP3, TRP4* and *TRP5* in being tightly linked to its centromere in *S. bayanus* as well as in *S. cerevisiae*, as indicated by synteny relationships (**http://db.yeastgenome.org/cgi-bin/FUNGI/FungiMap**; Kellis *et al*., 2003). Mutation to *trp1* in the haploid could result in recovery of the mutant. Mutation to *trp1* in a haploid spore still enclosed in the ascus of the homothallic strain would likely result in mating of the *trp1* spore to a wild-type spore, with loss of the FAA$^R$ Trp$^-$ phenotype. The same fate would likely overtake the *trp3, trp4* and *trp5* mutants as well. Possibly, however, there is sufficient residual growth of the heterozygous cells to allow mitotic recombination between the *trp3, trp4* and *trp5* mutations and their respective centromeres, resulting in homozygosis of these mutations. The proximity of *TRP1* to its centromere would preclude homozygosis of *trp1* mutations by this mechanism. This explanation seems unlikely, given the

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/yea

high frequency of mitotic recombination seemingly required. However, other explanations, such as differential mutability of *TRP1* in haploid vs. diploid cells, or an absence of intra-ascal mating until a delay in mating of *trp3, trp4* and *trp5* mutant spores and their daughters but not *trp1* mutant spores and their daughters through al least two mitoses (assuming these homothallic *Saccharomyces* species follow the same switching rules as *S. cerevisiae*) allows generation of $Trp^{-}$ spores of opposite mating type, seem even more far-fetched.

## Acknowledgements

We thank Tatyana Aleynikova for technical support. We thank the Howard Hughes Medical Institute for support of this summer undergraduate research programme by a Howard Hughes Medical Institute Professorship to E.W.J.

## References

Boeke JD, LaCroute F, Fink GR. 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet* **197**: 345–346.

Cliften PF, Hillier LW, Fulton L *et al*. 2001. Surveying *Saccharomyces* genomes to identify functional elements by comparative DNA sequence analysis. *Genome Res* **11**: 1175–1186.

Jones EW, Zubenko GS, Parker RR. 1982. *PEP4* gene function is required for expression of several vacuolar hydrolases in *Saccharomyces cerevisiae*. *Genetics* **102**(4): 665–677.

Kellis M, Patterson N, Endrizzi M, Birren B, Lander ES. 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* **423**: 241–254.

Naumov GI, James SA, Naumova ES, Louis EJ, Roberts IN. 2000. Three new species in the *Saccharomyces sensu stricto* complex: *Saccharomyces cariocanus, Saccharomyces kudriavzevii*, and *Saccharomyces mikatae*. *Int J Syst Evol Microbiol* **50**(5): 1931–1942.

Petersen RF, Nilsson-Tillgren T, Piskur J. 1999. Karyotypes of *Saccharomyces sensu lato* species. *Int J Syst Bacteriol* **4**: 1925–1931.

Toyn JH, Gunyuzlu PL, White WH, Thompson LA, Hollis GF. 2000. A counterselection for the tryptophan pathway in yeast: 5-fluoroanthranilic acid resistance. *Yeast* **16**: 553–560.

Zubenko GS, Park FJ, Jones EW. 1982. Genetic properties of mutations at the *PEP4* locus in *Saccharomyces cerevisiae*. *Genetics* **102**: 679–690.

Copyright © 2007 John Wiley & Sons, Ltd.


DOI: 10.1002/yea