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Recent Crop-To-Weed Adaptive Introgression Has Reshaped the Genomic Composition and Geographical Structure of US Weedy Rice (*Oryza* spp.)

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ABSTRACT

Weedy rice is a close relative of cultivated rice (*Oryza sativa*) that infests rice fields worldwide and drastically reduces yields. To combat this agricultural pest, rice farmers in the southern US began to grow herbicide-resistant (HR) rice cultivars in the early 2000s, which permitted the application of herbicides that selectively targeted weedy rice without harming the crop. The widespread adoption of HR rice coincided with increased reliance on hybrid rice cultivars in place of traditional inbred varieties. Although both cultivated and weedy rice are predominantly self-fertilising, the combined introductions of HR and hybrid rice dramatically altered the opportunities and selective pressure for crop-weed hybridization and adaptive introgression. In this study, we generated genotyping-by-sequencing data for 178 weedy rice samples collected from across the rice growing region of the southern US; these were analysed together with previously published rice and weedy rice genome sequences to determine the recent genomic and population genetic consequences of adaptive introgression and selection for herbicide resistance in US weedy rice populations. We find a reshaped geographical structure of southern US weedy rice as well as purging of crop-derived alleles in some weed strains of crop-weed hybrid origin. Furthermore, we uncover evidence that related weedy rice strains have made use of different genetic mechanisms to respond to selection. Lastly, we identify widespread presence of HR alleles in both hybrid-derived and nonadmixed samples, which further supports an overall picture of weedy rice evolution and adaptation through diverse genetic mechanisms.

1 | Introduction

Adaptive introgression is the multi-generational process by which a population hybridises with members of a genetically diverged group, backcrosses to members of its own group and eventually purges recombinant genotypes that are maladaptive (Barton 2001; Janzen, Wang, and Hufford 2019). This process leaves the population with a newly acquired adaptation that might have otherwise required multiple generations to evolve via random mutation. Although the first-generation effects of heterosis in F_1 hybrids are well studied (Labroo, Studer, and Rutkoski 2021), and the persistence of introgressed alleles has been well documented in the deep evolutionary history of species (e.g., Neanderthal introgression in the modern human lineage)

(Leroy et al. 2020; Pardo-Diaz et al. 2012; Racimo et al. 2015; Whitney, Randell, and Rieseberg 2010), rarely is adaptive introgression documented in the intermediate generations following a hybridization event and tracked on a contemporary time scale where the fates of hybrid genomes can be directly observed (Hedrick 2013; Oziolor et al. 2019).

Most descendants of F_1 hybrids between divergent groups are expected to be far from a fitness peak, resulting in low average fitness. The first alleles to rise in frequency in subsequent generations are predicted to be those at large-effect loci that are strongly favoured by selection, whereas alleles approaching fixation later will tend to be small-effect loci (Edelman and Mallet 2021). This leads to an interesting natural experiment where in the generations following a hybridization event, early selective sweeps can shed light on the most important regions of the genome for the survival and reproduction of the newly hybridised population.

In addition to the genomic effects of adaptive introgression on individual genomes, this process also has consequences at the population level (Hedrick 2013). A given hybridization event is a single episode that has a discreet geographic origin. Thus, the consequences of that event can be thought of as similar to a mutation in classic population genetic theory, whereby a new allele enters the population at low frequency and is exposed to evolutionary forces including selection, genetic drift and gene flow. If gene flow rates are low and selection is strong, it is possible that a previously unstructured population could transiently evolve a strong geographical structure through this process. Multiple such events across a species range would further enhance the emergence of population structure (Chhatre et al. 2018).

1.1 | Weedy Rice Evolution in the Southern US

Modern agricultural settings are useful environments for documenting recent and rapid evolution, including the process of adaptive introgression (Vigueira, Olsen, and Caicedo 2013). Agricultural weeds, which have evolved to exploit the agricultural habitat without intentional selection by humans, have proved to be especially useful study systems, providing important insights into the molecular basis and timing of adaptation to agroecosystems (Kreiner et al. 2019; Li and Olsen 2020; Van Etten et al. 2020; Wu, Lao, and Fan 2021). Those agricultural weeds that grow in sympatry with reproductively compatible crop relatives are particularly interesting for studies of adaptive introgression due to the potential for genetic exchange (Ellstrand, Prentice, and Hancock 1999).

Among weedy crop relatives, weedy rice (*Oryza spp.*), a close relative of the model crop species rice (*O. sativa*), has been the most extensively studied through genomic approaches. Weedy rice has evolved multiple times independently in rice-growing regions worldwide, primarily through de-domestication (endoferalization) of local crop varieties (Grimm et al. 2013; Qiu et al. 2020), but also through processes involving hybridization and adaptive introgression between diverse populations of cultivated, wild and weedy rice. Studies from South America, Southeast Asia, China and the United States have identified adaptive introgression as a major driver of weedy rice evolution (Li et al. 2024; Presotto et al. 2024; Sun et al. 2013; Wedger, Roma-Burgos, and Olsen 2022; Wedger et al. 2019).

In the major US rice-growing region of the southern Mississippi River valley, there were historically (i.e., before the 21st century) two morphologically and genetically distinct strains of weedy rice (Londo and Schaal 2007; Olsen, Caicedo, and Jia 2007; Reagon et al. 2010): Strawhull awnless (SH)—characterised by a short, crop-mimic like stature and straw-coloured seed hulls; and Blackhull awned (BHA)—characterised by tall plant architecture with purple culms, black seed hulls and long and barbed awns. These strains, which are most closely related to the Asian *indica* and *aus* domesticated varietal groups, respectively, are genetically distinct from the *tropical japonica* cultivars grown in the southern US (Reagon et al. 2010) and are thought to have evolved through de-domestication in Asia before their unintentional introduction as weeds into the US (Londo and Schaal 2007). As weeds directly descended from domesticated rice, the evolution of the SH and BHA strains occurred in part through the reemergence of traits found in wild *Oryzas* that were selected against during domestication, including seed-dispersal mechanisms (shattering) and the ability to remain viable for several years in the soil seed bank (seed dormancy). Due to the genetic differences between US cultivars and weedy rice, as well as a high degree of self-fertilisation in both weedy and cultivated rice, outcrossing rates between weedy and cultivated rice in the southern US were historically less than 1% (Shivrain et al. 2009). Additionally, while crop-weed hybrids did sometimes form, the newly formed hybrid lineage rarely survived, and advanced-generation hybrids were not detected in population surveys (Reagon et al. 2010). Before the 21st century, SH and BHA co-occurred in rice fields at similar frequencies, and surveys throughout the southern US rice belt indicated little evidence for within-strain geographical population structure (Reagon et al. 2010).

The evolution of SH and BHA weedy rice began to change radically beginning in the early 2000s as a result of two major technological changes in US rice agriculture (Figure 1b). In 2000, the first US hybrid rice cultivars were commercialised. While these hybrid genotypes were higher yielding than the traditionally planted inbred lines (due to heterosis), they were also prone to shattering mature seed in the field that could overwinter and re-emerge in subsequent years as 'volunteer rice' (Singh et al. 2016, 2017a). Moreover, as descendants of F_1 hybrids, trait segregation in the F_2 and subsequent generations of these volunteers created a broad range of phenotypes, including for traits associated with weediness (shattering, dormancy and high-competitive ability for light, nutrients and other resources); they were also characterised by a widened window of flowering time that maximised opportunities for hybridization with SH and/or BHA weedy rice. Thus, the widespread commercialization of hybrid rice in the US had two unintended consequences: the formation of a crop-to-weed gene flow bridge, and the spawning of a new form of weedy 'volunteer rice' that, while directly descended from US cultivars, shared many weedy characteristics of the SH and BHA weeds that were already present.

The second major innovation in 21st-century US rice agriculture was the introduction of herbicide-resistant (HR) Clearfield rice (BASF, CA, United States) in 2002, which contains mutations in

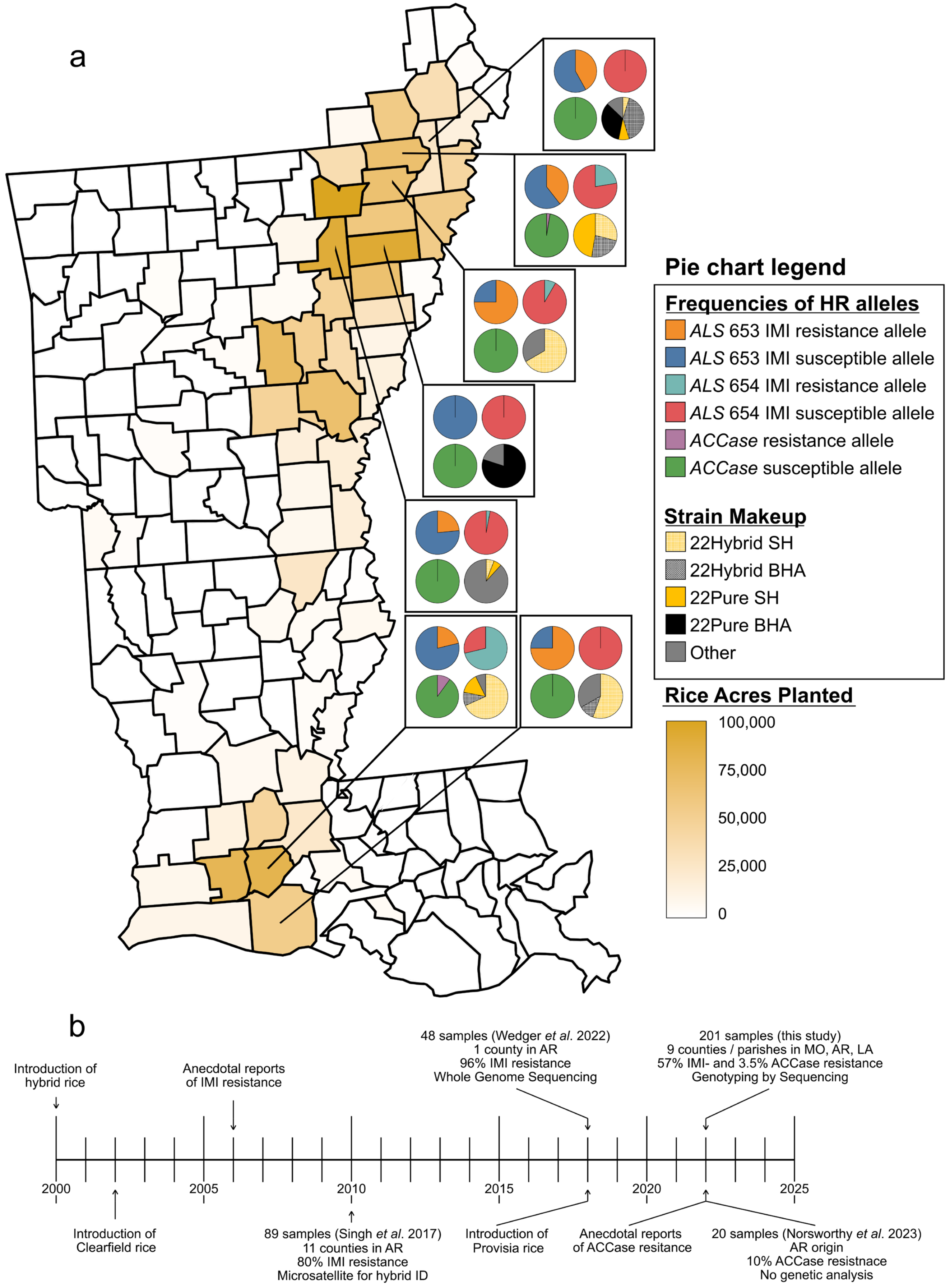

FIGURE 1 | Legend on next page.

FIGURE 1 | County and parish map of rice acres planted in 2021 as reported by USDA Farm Service Agency (a) and timeline visualisation of the evolution of herbicide resistance in weedy rice in the southern US (b). Higher planted acres of rice are shaded darker in yellow. Pie charts represent allele frequencies of *ALS* $S_{653}N$ (top left of each box), *ALS* $G_{654}E$ (top right of each box), *ACCase* (bottom left of each box). Bottom right pie chart of each box represents the strain makeup of each county with hatched-straw and hatched-black coloured sections representing 22Hybrid SH and 22Hybrid BHA, respectively, solid-straw and solid-black coloured sections representing 22Pure SH and 22Pure BHA, respectively, and grey representing an "other" category that includes 22Hybrid Weed, 22Triple Hybrid and 22Volunteer samples.

the *Acetolactate synthase (ALS)* gene conferring resistance to the imidazolinone (IMI) family of herbicides. In IMI-resistant rice plants, two possible *ALS* mutations, $S_{653}N$ and $G_{654}E$ (numbering based on *Arabidopsis thaliana* amino acid sequence (Sales et al. 2008)), prevent IMI herbicides from disrupting biosynthesis of branched amino acids that are essential to the plant's survival (Rajguru et al. 2005). This technology allowed farmers to target weedy rice with IMI herbicides while keeping the crop safe. By the mid-2000s, Clearfield cultivars that were also hybrid genotypes were introduced; these delivered high yields with remarkably low infestation rates. Adoption of these technologies was quick, with hybrid rice rising to 50% of fields over a 20-year period, while Clearfield utilisation peaked at 65% in the mid-2010s (Moldenhauer, Scott, and Hardke 2020). With the manufacturer-recommended regime of crop rotation to mitigate HR weed emergence, most southern US rice fields were planted with Clearfield cultivars at least once every 3 years (Clearfield, soybean, then non-HR rice rotations in AR and MO; Clearfield, crawfish aquaculture, then non-HR rice rotations in LA).

Only 4 years after the introduction of Clearfield HR rice in 2002, farmers began to anecdotally report HR weedy rice. Shortly thereafter, these reports were verified through genetic analysis (Rajguru et al. 2005). Population sampling from 2006 onward uncovered increasing proportions of weedy rice populations gaining IMI resistance (Singh et al. 2017a, 2017b), with our most recent collection in 2018 identifying 98% of samples from the primary rice-growing region of Arkansas with IMI resistance alleles (Wedger, Roma-Burgos, and Olsen 2022). The large majority (92%) of these 2018 samples were determined to be of crop-weed hybrid descent, containing the crop-specific haplotype at *ALS*, while a few (8%) contained a mutation identical to one of the Clearfield resistance mutations but without genomic evidence of crop-weed hybridization. Thus, as of 2018, genetic analyses suggested that there was overwhelming selection for the IMI-resistant allele in southern US weedy rice, and that adaptive introgression from HR cultivars explained the majority of HR genotypes, but with some resistance alleles apparently of non-crop origin (reflecting *de novo* evolution and/or standing variation) in weed populations.

Further findings of our 2018 study were that (1) BHA-derived crop-weed hybrids far outnumbered SH-derived hybrids (by a 3.7: 1 ratio); (2) hybrid-derived weed genomes were composed of majority (~70%) weedy rice local ancestry across their genomes; (3) no 'genomically pure' weeds of BHA background were detected, suggesting they may have been entirely displaced by BHA-crop hybrid derivatives; and (4) all weeds with resistance alleles but without evidence of cultivar introgression were SH genotypes. However, because the 2018 sampling of weedy rice focused specifically on the region of highest rice production (eastern Arkansas), the generalizability of these findings for the larger southern US rice belt remained unknown. Another knowledge gap has been whether US weedy rice is still characterised by a lack of geographical population structure, as observed for pre-2000s populations, or whether this has been altered by crop-weed hybridization events. Moreover, given the speed at which the genomic composition of US weedy rice has been altered within the last 20 years, an additional unanswered question is how hybrid weedy rice genomes have continued to evolve in the rapidly changing rice agroecosystem.

In the years since our 2018 collections in Arkansas, rice farmers have reported that the Clearfield technology has largely failed due to high levels of IMI resistance in weedy rice (Butts et al. 2022; Roma-Burgos et al. 2021). To fill the gap left by Clearfield's waning effectiveness, a second HR cultivar was commercialised in 2018. Provisia (BASF, CA, United States) rice is resistant to the acetyl coenzyme-A carboxylase (ACCase) inhibitor quizalofop-p-ethyl (QPE), which prevents the synthesis of fatty acids and is lethal to susceptible plants. Resistance to QPE is gained via an A-to-T single nucleotide substitution in the *ACCase* gene that results in a $L_{1781}I$ replacement (Camacho et al. 2019). Shortly after the release of Provisia, a second QPE-resistant cultivar was also commercialised under the name Max-Ace (RiceTec Inc., TX, United States). The Max-Ace mutation remains proprietary as of this date; however, Max-Ace cultivars have a lower tolerance to QPE than Provisia rice, suggesting different underlying mutations (González-Torralva and Norsworthy 2023). Just 4 years after the introduction of QPE-resistant rice, farmers again began to share anecdotal evidence of the emergence of HR weedy rice, and this was quickly confirmed via QPE-resistance screens and *ACCase* gene sequencing which identified the Provisia allele in weedy rice samples but could not confirm crop-weed hybridization (González-Torralva and Norsworthy 2023). It is unknown how the recent widespread shift away from complete reliance on IMI-resistant cultivars has affected the evolution of weedy rice genotypes.

In this study, we sought to answer five primary questions related to the recent evolution and adaptation of US weedy rice: (1) How do post-2018 collections of weedy rice (specifically, genotypes sampled in 2022 from throughout the southern US rice belt) compare to the 2018 Arkansas collections in terms of the relative proportions of 'genomically pure' vs. hybrid-derived SH and BHA weed strains? (2) Do the genomes of hybrid-derived weeds indicate an over-representation of weed-like genomic components consistent with purging of crop alleles, as was previously detected? (3) Is there evidence of selective sweeps in contemporary weedy rice genomes; if so, what can they tell us about the importance of HR loci vs. other genomic regions as critical targets of selection in contemporary weed populations? (4) Is adaptive introgression still the primary method of IMI-resistant evolution in Arkansas, and is the same mechanism observed throughout the southern US rice belt? (5) How has weedy rice responded to the introduction of Provisia HR rice, and how widespread is resistance to this recently deployed HR technology?

2 | Materials and Methods

2.1 | Sampling and DNA Preparation

In the summer of 2022, whole panicles from individual weedy rice plants were collected from 18 rice fields across 9 counties or parishes in 3 states for a total of 201 samples (Table S1). All samples taken from a single field were given the same letter designation and sequentially numbered. Samples were collected from at least 10 m apart to minimise collection of full siblings or other close relatives. A single seed from each panicle was grown in the Washington University in St. Louis greenhouse in ambient greenhouse conditions until the seedling stage, when leaf tissue was harvested for DNA extraction and subsequent library preparation and polymerase chain reaction (PCR) genotyping. Tissue (0.6 g) was ground in liquid nitrogen, and genomic DNA was extracted using a DNeasy Plant kit (Qiagen, Hilden, Germany) following the included protocols. Additional genomic data were used from existing sources; these included whole genome sequences of 48 weedy rice samples from our 2018 study (Wedger, Roma-Burgos, and Olsen 2022), 22 historic weedy rice samples from collections made in the 1990s, before the commercialization of HR or hybrid rice, which are the same representatives as used in the previous study (Li et al. 2017), and 34 cultivated rice samples from a recently published pan-genome analysis (Shang et al. 2022). Cultivated samples included *indica* ($n=10$), *japonica* ($n=20$) and *aus* ($n=4$). Samples were randomly chosen as a subset of each genetically distinct group to maximise the phylogenetic breadth of samples as analysed by Shang et al. (2022).

2.2 | GBS Library Preparation and SNP Genotyping

Genotyping-by-sequencing (GBS) sequencing libraries were prepared from extracted DNA following established protocols (Wright et al. 2022). In short, genomic DNA was digested using the ApeKI restriction enzyme, prepped with the P5/P7 protocol, and size selected with 0.8× magnetic beads. Successful libraries were multiplexed and sent to Novogene (Novogene Corporation Inc., Sacramento, CA) for sequencing. Raw Illumina reads from previously published genome sequences were obtained from NCBI GenBank. Upon data return, raw reads were demultiplexed, trimmed, aligned, and converted to .bam files using the *SABRE v1.00*, *cutadapt v1.18*, *bwa v0.7.18* and *samtools v1.15* packages, respectively, as part of the *fastgbs_V2 pipeline v.1.0* (Torkamaneh, Laroche, and Belzile 2020).

The *GATK v4.5.0.0* pipeline (HaplotypeCaller, GenomicsDBImport and GenotypeGVCFs) was used for SNP (Single Nucleotide Polymorphism) calling and VCF (Variant Call Format) file creation (McKenna et al. 2010). GATK imputes reference alleles for cites with zero coverage, so zero coverage cites were converted to missing data with the *BCFtools v1.14* + setGT command. VCFs were initially created for GBS and whole-genome samples separately. THE GBS-derived VCF file was filtered with the following flags in *vcftools v0.1.16* (Danecek et al. 2011): --remove-indels, --max-alleles 2, --hwe 0.0000000001, and --max-missing 0.95 to obtain a list of genotyping-by-sequencing (GBS)-specific SNPs. This list was then used to filter the whole-genome VCF using the --keep flag in *vcftools*. Filtered VCFs were merged using the merge command in *BCFtools v1.14* (Danecek et al. 2021) and filtered with vcftools again with the addition of the --maf 0.05 flag.

2.3 | Population Structure and Principal Component Analysis

ADMIXTURE v1.3.0 (Alexander, Novembre, and Lange 2009) and PCA in *Plink1.9* (Purcell et al. 2007) were performed with the combined dataset to establish genetic relationships between newly collected and previously analysed samples. *ADMIXTURE* results were visualised with *Pong v1.5* (Behr et al. 2016). Because the potential ancestors of southern US weedy rice hybrids are known, *ADMIXTURE* was run as a supervised analysis with *tropical japonica* rice (the varietal group to which southern US crop cultivars belong), historic (pre-2000) BHA weedy rice, and historic (pre-2000) SH weedy rice as defined population groups. For comparison, it was also run as an unsupervised analysis. Unknown samples were assigned ancestry based on *ADMIXTURE* analysis. Samples were categorised in line with Wedger, Roma-Burgos, and Olsen (2022), such that those with BHA ancestry were categorised as 'BHA-like', samples with SH ancestry were categorised as 'SH-like', and samples with three or more ancestral representatives were categorised as 'complex'. Weedy rice samples collected in 2018 were grouped by their previous designation from Wedger, Roma-Burgos, and Olsen (2022) and were kept separate from samples collected in 2022 for visual comparison.

2.4 | Genome-Wide Local Ancestry and Selection Scans

Local ancestry across the genome was calculated using the *Loter v1.0* software (Dias-Alves, Mairal, and Blum 2018) which analyzes VCF files for two proposed ancestor populations and a third VCF of hybrid-derived samples. *Loter* output was used to quantify the proportion of ancestral genomes that made up contemporary hybrid samples and to visualise relative ancestor contributions across the genomes of hybrid derivatives. Visualisations were performed with *Matplotlib v3.9.2* (Hunter 2007). *Loter* was also used to investigate fine-scale local ancestry at the gene of interest, *ALS*, by comparing local ancestry of the surrounding 200 kb region to randomly selected 200 kb regions of the genome using a custom *Python3* script.

Genome-wide selection scans were performed using two methods: *XPCLR v1.0*, which identifies outlier regions of genetic differentiation between population groups (Chen, Patterson, and Reich 2010); and *SweeD4.0.0* with a grid size of 20,000, which identifies genomic regions of positive selective sweeps within a single group using site frequency spectra (Pavlidis et al. 2013). The top 1% of windows from *XPCLR* and top 1% of loci from *SweeD* were considered candidate selective sweep regions. As *XPCLR* is based on population differentiation, the analysis was performed by comparing a focal weedy rice group with its closest presumed ancestors, specifically by comparing *aus* to historic BHA and historic BHA to 22Hybrid BHA. As our focus was on how genomes have evolved since the agricultural changes of the 21st century, any selection window that appeared in both sets was discarded as reflecting weed evolution before the focal time period. Overlap between *XPCLR* and *SweeD* windows was identified as shared windows. Analysis for the SH groups was identical to those used for BHA but compared *indica* to historic SH and historic SH to 22Hybrid SH. *XPCLR* and *SweeD* results were plotted with r/qqman. GO (Gene Ontology) overrepresentation analysis was performed by returning genes within *XPCLR* windows as .bed files using the *Phytozome 13* genome browser (https://phytozome-next.jgi.doe.gov/) and the *g:Profiler* (https://biit.cs.ut.ee/gprofiler/gost) online tool with the *Oryza sativa indica group* annotation. BHA-only, SH-only and shared GO overrepresentation analyses were conducted separately.

2.5 | PCR Genotyping of Herbicide-Resistance Genes

Primer sets for HR allele genotyping were obtained and optimised from previous studies to identify the presence of alleles responsible for herbicide resistance to imidizolinone at the gene *ALS* (Kadaru et al. 2008) and QPE at the gene *ACCase* (Camacho et al. 2019). There are two major resistance alleles at *ALS* (Sales et al. 2008), $\text{Ser}_{653}\text{Asn}$ ($S_{653}N$) and $\text{Gly}_{654}\text{Glu}$ ($G_{654}E$), so a resistant and susceptible primer set were optimised for both resistance alleles (Table S2). Thermocycler conditions for the six primer sets were as follows: ALSG654RresF, ALSG654RsusF, ALSS653DresF and ALSG653RsusF all shared reverse primer ALSR3. The ALSG654RresF, ALSG654RsusF and ALSS653DresF primer sets were initially denatured at 95°C for 4 min followed by 28 cycles of denature at 95°C for 30 s, annealing at 51°C for 30 s and extension at 72°C for 30 s. The final extension was 72°C for 5 min. The ALSG653RsusF primer set was similar, but the annealing temperature was reduced to 48°C. OsACCp1A-F with OsACCp1A-R and OsACCp1B-F with OsACCp1B-R primer sets were initially denatured at 95°C for 3 min followed by 30 cycles of denature at 95°C for 20 s, annealing at 60°C for 20 s and extension at 72°C for 20 s. The final extension was 72°C for 3 min. All primer sets were designed such that primers were allele-specific via differential annealing to template DNA based on the identity of the focal SNP (resulting in fragment size variation visualizable on a 2% agarose gel).

3 | Results

Seeds from 201 panicles were collected from 18 fields across 9 counties or parishes in 3 states during the 2022 harvest season, which ranged from mid-August in Louisiana to late-September in the Bootheel of Missouri (Figure 1a). GBS data were successfully obtained for 178 samples using the ApeKI restriction enzyme and combined with previously published whole-genome data from three sources (Table S1). This resulted in a final dataset of 282 individuals: 178 samples collected for this study, 48 weedy rice samples collected in Arkansas in 2018, 34 cultivated rice samples published in a recent pan-genome study (4 *aus*, 10 *indica* and 20 *tropical japonica*) and 22 weedy rice samples collected from before the agricultural advances of the early 2000s that introduced hybrid and herbicide-tolerant rice (12 historic BHA and 10 historic SH). After filtering to $<5\%$ missing data, the resulting SNP dataset included 30,487 SNPs.

3.1 | Population Genomic Composition of US Weedy Rice Has Been Altered From Historic Patterns

To assess the composition of contemporary weedy rice across the southern US rice growing region, we performed supervised *ADMIXTURE* and principal component analysis (PCA). The supervised *ADMIXTURE* analysis revealed seven distinct genetic sub-groups in our 2022 collections (Figure 2a). First, samples with primarily BHA ancestry (broadly categorised as 'BHA-like') fell into two groups: crop-weed hybrid BHAs ($>10\%$ ancestry from crop and BHA, designated '22Hybrid BHA', $n=39$) and 'genomically pure' (nonadmixed) BHAs ($>90\%$ ancestry from BHA, designated '22Pure BHA', $n=25$). Samples with primarily SH ancestry ('SH-like') similarly fell into two groups: crop-weed hybrid SHs ($>10\%$ ancestry from crop and SH, designated '22Hybrid SH', $n=52$) and 'genomically pure' (nonadmixed) SHs ($>90\%$ ancestry from SH, designated '22Pure SH', $n=30$). The fifth and sixth groups included samples with complex admixed ancestry, such as those with high ancestry from SH, BHA and cultivated rice collectively, which could only arise through multiple rounds of hybridization ($>10\%$ ancestry from SH, BHA and crop, designated '22Triple hybrid', $n=10$), and those with admixture of SH and BHA ancestry without detectable crop ancestry ($>10\%$ ancestry from SH and BHA, designated '22Hybrid weed', $n=9$). The final group identified by *ADMIXTURE* were those with no historic weedy rice (SH or BHA) ancestry. As these plants were visually identified and collected as weeds, they were considered volunteer descendants of crop cultivars ($>90\%$ ancestry from crop, designated '22Volunteer', $n=13$). An unsupervised *ADMIXTURE* was also performed with comparable results (Figure S1).

The PCA recapitulated patterns found in our previous study based on whole genome sequencing (Wedger, Roma-Burgos, and Olsen 2022). Importantly, this indicates that the reduced representation GBS SNP data set of the present study provides sufficient resolution to characterise population genetic structure (Figure 2b). PC1 (33.5% of variance explained) largely separates on an *indica-aus* axis, while PC2 (18.5% of variance explained) is better explained as an *indica-japonica* axis. The PCA showed patterns in the 2022 samples similar to those found in 2018, such as 22BHA-like samples separated from 22SH-like samples along PC1, with *tropical japonica* samples intermediate between them. This is consistent with two hybrid populations that share a single ancestor. Furthermore, 2022 crop-weed hybrid samples were intermediate between proposed crop and weed ancestors, supporting the validity of ADMIXTURE-informed identifications. Finally, 22Volunteer samples clustered tightly with *tropical japonica*, while 22Hybrid weed and 22Triple Hybrid samples were intermediate between their proposed ancestors as well. Taken together, the PCA and supervised *ADMIXTURE* analyses provide evidence consistent with the introgression of cultivated rice genotypes into weedy rice but also the persistence of 'genomically pure' SH and BHA populations around the region.

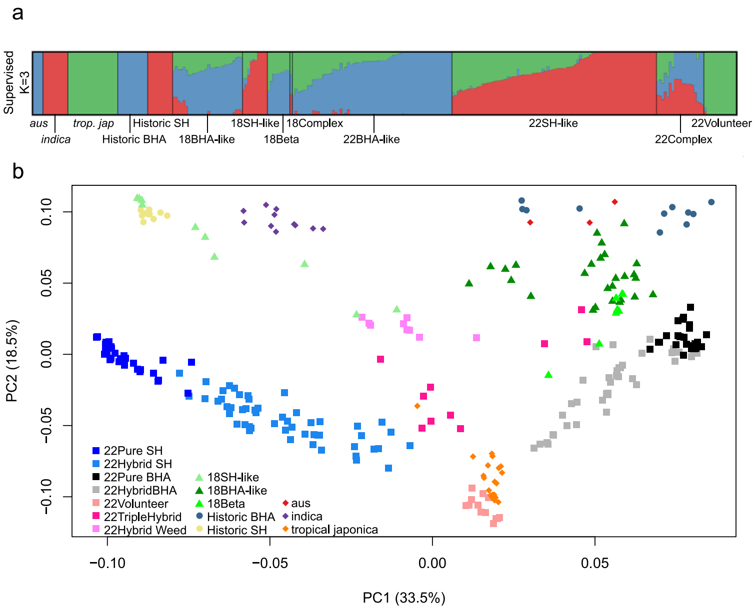

FIGURE 2 | Supervised *ADMIXTURE* (a) and PCA (b) plots for all samples analysed in this study. *ADMIXTURE* was supervised with three populations: Historic BHA, Historic SH and *tropical japonica* (*trop. jap*). 22BHA-like includes 22Hybrid BHA and 22Pure BHA. 22SH-like includes 22Hybrid SH and 22Pure SH. 22Complex includes 22Hybrid Weed and 22Triple Hybrid. In the PCA square, triangle, circle and diamond markers in PCA denote 2022 collections, 2018 collections, pre-2000 collections and cultivated rice, respectively.

Geographical heterogeneity in genotype distributions was evident at the level of individual fields, with fields within the same county having distinct weed profiles (Figure 1a, Table S1). For example, in Jefferson County, AR, field "T" was made up of entirely 22Volunteer weeds, while field "L" contained primarily 22Hybrid Weed (SH-BHA hybrids) samples. Similarly, field "Y" in Dunklin County, MO, had exclusively 22Hybrid BHA weeds, while Field "X" contained almost exclusively 22Pure BHA weedy rice despite their proximity within a single county. These patterns indicate a degree of geographical structure in southern US weedy rice populations that has not previously been reported.

3.2 | Region-Wide Local Ancestry Measures Support Post-Hybridization Evolution Towards Weedy Ancestry

Loter (Dias-Alves, Mairal, and Blum 2018) is a local ancestry analysis tool that assigns haplotype blocks of hybrid genomes to potential ancestors under the assumption of a two-ancestor model. For this analysis, 2022 samples were assigned to potential weedy ancestors based on assigned ancestry in the *ADMIXTURE* analysis; they were assumed to have *tropical japonica* crop ancestry if identified as crop-weed hybrids. (Samples belonging to the 22Hybrid weed and 22Triple hybrid groups were excluded from *Loter* analysis due to inappropriate assumption of crop ancestry for the former, and inappropriate assumption of dual ancestry in the latter.) 22Hybrid BHA showed a genome-wide level of weedy rice ancestry (~60%) that was comparable to collections in 2018 (Figure 3a); this suggests a continuing overall selective advantage of weedy over crop haplotypes across the genome in BHA-derived weedy rice. In contrast, the region-wide collections showed significantly less weedy rice ancestry in 22Hybrid SH genomes than in earlier SH hybrid collections, with contemporary genomes nearly evenly split (~50%) between crop and weedy ancestry ($p = 0.03$, 2-tailed unpaired Student's t-test with equal variance). For purposes of comparison, groups without evidence of hybrid ancestry in the ADMIXTURE and PCA analyses were also subjected to *Loter* analysis (Figure S2). Consistent with their lack of US crop ancestry, samples of 22Pure BHA and 22Pure SH indicated predominant weedy rice ancestry; also consistent with expectations, the 22Volunteer samples, which lack SH or BHA ancestry, indicated minimal weedy rice ancestry.

Fine-scale analysis of *Loter* scores in the 200 kb region surrounding the *ALS* herbicide resistance gene revealed that 22Hybrid BHA and 22Hybrid SH populations show a bias for the cultivated genome when compared to randomly selected ~200 kb regions (Figure 3b). In both populations, average *Loter* scores in the *ALS* genomic region were more than one standard deviation lower than the mean (i.e., more crop-like), suggesting that while genome-wide *Loter* scores indicate a bias for the weedy ancestor, this gene region shows the opposite pattern with a bias toward crop ancestry. Our estimate of *Loter* scores at *ALS* are also likely conservative in estimating crop ancestry as related to resistance allele introgression, since many hybrid samples (36/76) do not contain resistance alleles (Table S1). Visually, the skew of random windows also recapitulates our inferences from genome-wide estimates of *Loter* scores (Figure 3b). In 22Hybrid BHA samples, the average random window is above 0.5, suggesting a weed-like genomic bias, while in 22Hybrid SH samples the normal distribution of random windows is centered at 0.5, with an excess of slightly crop-like windows pulling the average below 0.5.

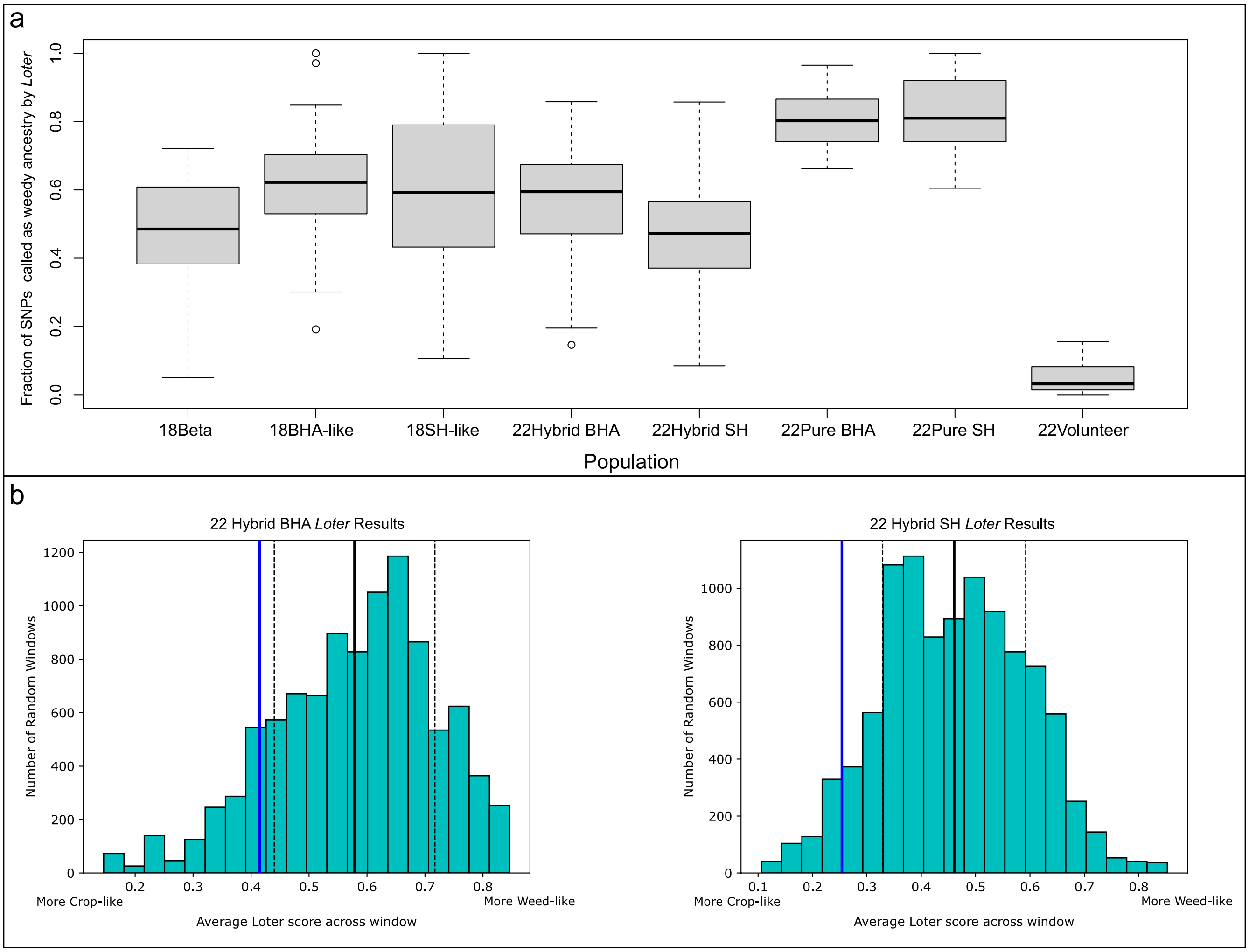

FIGURE 3 | Box-and-whisker plot of local ancestry estimations based on *Loter* analysis (a) and fine scale analysis of local ancestry around *ALS* (b). In panel a, data are plotted as the fraction of SNPs within our dataset that were called as having weedy ancestry. 18BHA-like and 22Hybrid BHA are not significantly different from each other ($p=0.13$, 2-tailed unpaired Student's t-test with equal variance), while 18SH-like and 22Hybrid SH are significantly different from each other ($p=0.03$, 2-tailed unpaired Student's t-test with equal variance). Whiskers represent maximum and minimum values after excluding outliers. Bounds of box represent first and third quartiles of data while thick black line represents the Median. In panel b, average *Loter* scores of 10,000 randomly selected 200 kb windows are presented as histograms, with higher values indicating more weed-like ancestry. Solid black vertical line indicates the population mean with one standard deviation above and below the mean shown as dotted black lines. Solid blue vertical line is the average *Loter* score of the population in the 200 kb window around *ALS*.

3.3 | Selection Scans Reveal Genomic Regions of Interest for 21st Century Weedy Rice Evolution

Genome-wide selection scans were performed by two complementary approaches. *XPCLR* is a differentiation-based method that identifies selective sweeps via allele frequency differentiation between two populations (Chen, Patterson, and Reich 2010), while *SweeD* detects selective sweeps via deviations in site frequency spectra within a population (Pavlidis et al. 2013). The top 1% of *XPCLR* 200 kb windows returned 186 individual windows. After collapsing consecutive outlier windows into single wider windows, we identified 49 and 47 windows for 22Hybrid SH and 22Hybrid BHA samples, respectively (Figure 4a,b). Notably, there were nine overlapping windows between 22Hybrid SH and 22Hybrid BHA *XPCLR* windows, suggesting a small amount of repeatability in the evolutionary responses to similar selection pressures in the two independently evolved weed lineages. *SweeD*, with a grid size parameter of 20,000 (suggested by the developer) returned many more candidate loci than XPCLR. Windows identified by *SweeD* varied greatly in size with a median of 2.27 Mbp. After filtering to the top 1% of loci and collapsing overlapping windows, *SweeD* identified 1480 windows in the 22Hybrid SHs, but only 98 in the 22Hybrid BHAs. This difference could suggest a difference in response to selection, with 22Hybrid BHA samples involving selection at fewer large-effect loci, and 22Hybrid SH plants involving selection on many small-effect loci.

Unexpectedly, we did not find any windows overlapping with the *ALS* HR gene in *XPCLR* analysis, despite the high likelihood that it is a target of strong selection. To investigate this further, we assessed SNP density across the region; this revealed a number of SNPs that was below the threshold required by *XPCLR* to detect a sweep (a minimum of at least five SNPs in a given 200 kb window). Thus, it appears that the genomic region around *ALS*

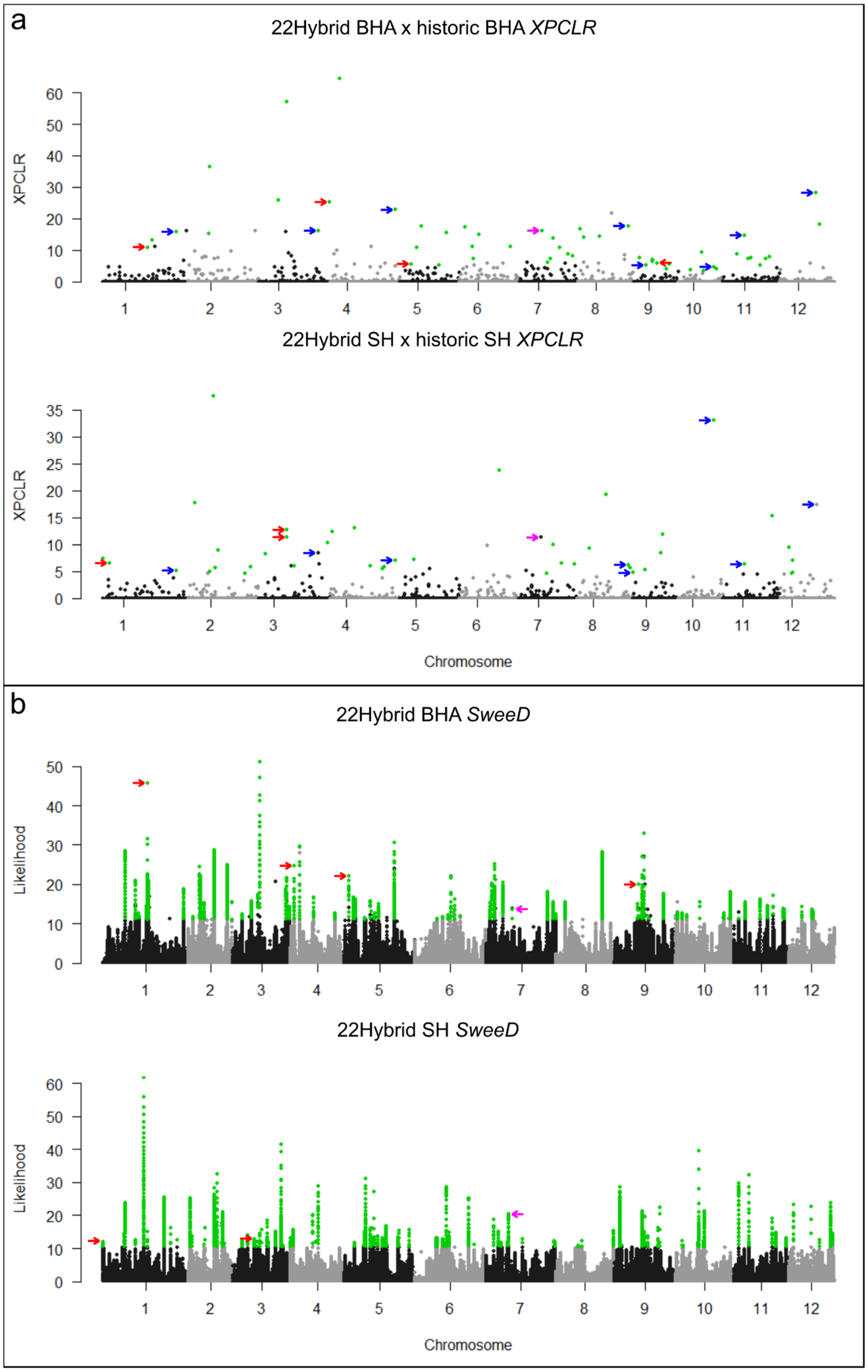

FIGURE 4 | Manhattan plots showing *XPCLR* (a) and *SweeD* (b) results. In panel a, green dots indicate top 1% of windows in contemporary × historic weedy rice *XPCLR* analysis after removing windows that also appeared in cultivated ancestor × historic weedy rice *XPCLR* analysis. Blue arrows indicate overlapping windows between strains within *XPCLR*. For both panels, red arrows indicate overlapping windows within strains and between statistical tests, and the single pink arrow indicates the sole window that is present in both strains and in both statistical tests. In panel b, green dots represent top 1% of windows in contemporary weedy rice *SweeD* analysis with windows that also appeared in historic weedy rice *SweeD* analysis removed. Blue arrows are not shown in panel b for visual clarity, but their genomic locations can be found in Table S2.

was likely not identified due to insufficient SNP density in the reduced representation GBS SNP dataset.

In comparisons between weed strains, 35 *SweeD* windows overlapped between the 22Hybrid SH and 22Hybrid BHA groups (Table S2), potentially highlighting important regions of the genome for weedy rice evolution. Additionally, four and five windows were shared by both XPCLR and *SweeD* in the 22Hybrid SH and 22Hybrid BHA weeds respectively. These windows are of particular interest for future candidate gene studies, as they reflect genomic regions, identified by two complementary approaches, that are involved in weedy rice evolution since the 21st-century agricultural advancements. Interestingly, a single window (chr7:11100001..17180000) appeared in the top

1% of results for both populations and both sweep analyses, potentially suggesting its functional importance for weed adaptation (although the window also appeared in the *indica*-historic SH analysis and was thus one of the discarded loci). This locus should be investigated in future studies on weedy rice adaptation. GO overrepresentation analysis by *g:Profiler* revealed a number of genes involved in Oxygen binding (GO:0019825) and Oxygen carrier activity (GO:0005344) for both the set of genes underlying 22Hybrid SH *XPCLR* windows and the set of genes underlying the nine windows identified by both selection scan approaches.

3.4 | HR Alleles Are Not Restricted to Hybrid-Derived Weeds

Through PCR-genotyping for HR mutations (Table S3), we identified 103/172 (60%) of successfully genotyped samples as carrying alleles conferring resistance to IMI herbicides (Figure 1a, Table S1). IMI-resistant genotypes were present at high frequency in Louisiana (44/49, 90.0%) and at moderate frequency across Arkansas and Missouri (34/66, 51.5% and 26/62, 42.0% respectively). Two mutations are known to confer resistance to IMI herbicides. Most IMI resistance-conferring genotypes in the 2022 collections carried the $S_{653}N$ allele (87/103 or 84.5%). The $G_{654}E$ resistance allele was present in 19/103 of the genotypes (18.4%). Interestingly, four samples (2.9%) carried both IMI resistance alleles. One of these individuals (LA_G04) was homozygous for both alleles, another (LA_H08) was heterozygous for $G_{654}E$ and homozygous for $S_{653}N$, the third (LA_H12) was homozygous for $G_{654}E$ and heterozygous for $S_{653}N$, and the last (AR_Q11) was heterozygous for both alleles. Based on a literature search, this is, to our knowledge, the first report of both alleles occurring within individual weedy rice genotypes. Notably, these four instances of dual IMI resistance alleles come from three unique fields spanning two states, suggesting they have independent origins. All samples from Greene County, Arkansas, had IMI-resistant genotypes, which is consistent with our previous results that focused specifically on this county (Wedger, Roma-Burgos, and Olsen 2022).

In our 2018 study, we found evidence for *de novo* evolution of both the $S_{653}N$ and $G_{654}E$ IMI-resistant alleles in weedy rice of non-hybrid ancestry. We found similar results here, with 19/25 (76%) of 22Pure BHAs having one of the two resistance alleles. Similarly, 19/24 (79.2%) of 22Pure SHs had an IMI-resistant allele. The converse was also true: several hybrid plants no longer carried an IMI-resistant allele, with 7/34 (20.6%) of 22Hybrid BHA and 29/42 (69%) of 22Hybrid SH samples lacking either allele. This result is consistent with a decrease in ongoing strong selection, potentially indicating a cost to maintaining the HR allele; however, this implication would need to be confirmed by fitness assessments.

QPE-resistant genotypes appeared to be much more rare than IMI-resistant alleles in the weedy rice samples. Only six individuals across 173 genotyped samples (3.5%) contained the crop QPE-resistant allele identified as responsible for resistance in Provisia cultivated rice (Camacho et al. 2019). The geographic range of this allele was also very limited, with five of the six weedy rice samples with the QPE resistance allele restricted to just one field in Louisiana (Table S1). Outside of this one field, only a single heterozygous individual was found in Arkansas. To our knowledge, although QPE-resistant weedy rice has been detected in herbicide screenings (González-Torralva and Norsworthy 2023), this is the first report of genetic evidence for the presence of this allele in weedy populations with a crop-weed hybrid origin, as each sample with this allele was identified as a crop-weed hybrid (see Table S1).

4 | Discussion

The conclusions derived from our 2018 whole-genome sequence analyses of weedy rice in Arkansas raised a number of follow-up questions, which we addressed in the present study. First, we assessed the generalizability of our previous study by broadening our geographical sampling beyond eastern Arkansas. Second, we used local ancestry analysis to estimate the genomic contribution of weedy and cultivated rice on contemporary hybrids. Third, we used selection scans to identify regions of the weedy rice genome that respond similarly to selection pressure despite divergent ancestries. Fourth, we used the presence of known herbicide-resistant alleles, combined with *ADMIXTURE* analysis, to determine the relative importance of crop-to-weed introgression as a driver in the evolution of herbicide resistance. Finally, we used a PCR-based approach to identify early instances of potential HR allele escape in the Provisia cropping system via putative crop-weedy hybridization.

In our geographically expanded analysis, we find evidence for a newly emergent geographical structure of weedy rice in the southern US; previous population genetic analyses had indicated a lack of geographical genetic structure in pre-2000s SH and BHA weed strains (Reagon et al. 2010). Given the short time since the evolution of HR in weedy rice, and the geographical distance between the sampled Missouri and Louisiana field sites, it is unlikely that hybrid-derived weeds of similar genomic composition arose via a single hybridization event and subsequently spread through long-distance dispersal; for example, 22Hybrid SH plants in Louisiana would be unlikely to represent migrants from 22Hybrid SH populations in northern Arkansas or vice versa. Combined with the field-specific strain compositions and the lack of clinal variation in strain makeup (Figure 1a, Table S1), it thus appears likely that crop-weed hybridization has, at least transiently, produced multiple independently evolving subpopulations. In the context of 21st-century agricultural practices, it seems that the adaptive introgression of IMI resistance alleles from cultivated to weedy populations was one of the main catalysts for this change. In other natural plant systems, interspecies adaptive introgression in response to environmental challenges has led to local adaptation and persistent population structure (Leroy et al. 2020; Rendón-Anaya et al. 2021); depending on weedy rice dispersal rates and the extent of geographical heterogeneity in selective pressures, it is possible that such patterns could become established in southern US weedy rice as well.

The risk of crop-to-weed or crop-to-wild gene escape through hybridization is an active research topic in agricultural science (Campbell et al. 2016; Knispel et al. 2008; Song et al. 2021; Warwick et al. 2008). Our results here can offer some insights into the genomic consequences on the recipients of crop-mediated hybridization. We find, among multiple independent evolutions of crop-weed hybrid sub-populations, that regardless of weedy rice strain ancestry (SH or BHA), genomes of hybrid-derived contemporary weedy rice are consistently biased against crop ancestry. This pattern compares to the expectation of a 50:50 crop-weed genomic composition of an initial F_1 hybrid. Under drift alone, subsequent generations would be expected to diverge from this ratio in both directions or not at all. The consistent reduction in crop ancestry observed here aligns with our previous approximations in weedy rice (Wedger, Roma-Burgos, and Olsen 2022) as well as in crop-wild sunflower hybrids (Corbi et al. 2018), suggesting a selective disadvantage for a high proportion of crop alleles across the genome in these non-cultivated populations.

The existence of two independently evolving weedy rice strains that coexist in rice fields, and which therefore experience similar selection pressures, allows us to ask questions on the extent to which separately evolving populations utilise similar or different underlying genetic mechanisms to maximise fitness. Our selective sweep scans, focused here on crop-weed hybrid descendants, add to the growing body of literature suggesting that the two weedy rice strains in the southern US use very different genetic mechanisms to respond to selection (Goad et al. 2020; Li et al. 2017; Qi et al. 2015; Wedger, Roma-Burgos, and Olsen 2022; Wedger, Topp, and Olsen 2019). These results can also likely be extended to other regions of the world where independently evolving strains of weedy rice are present (reviewed in Wedger and Olsen 2018). It should be noted, however, that our selective sweep inferences are based on a reduced representation SNP dataset. The inability of our dataset to identify *ALS* due to insufficient coverage suggests that while GBS sequencing is adequate for broad population genetic analyses, whole-genome sequencing is still required for in-depth genome scans to comprehensively identify selective sweeps.

Our region-wide results in the present study refine the conclusions of our 2018 study regarding the relative importance of crop-to-weed adaptive introgression vs. the evolution of herbicide resistance without gene flow from HR cultivars in contemporary weedy rice. In our 2018 Arkansas samples, we found that the vast majority (44/48) of samples were of crop-weed hybrid origin and contained the cultivated haplotype at *ALS*. We concluded from those results that adaptive introgression was the primary, but not sole, driver of weedy rice evolution since the 21st-century agricultural changes. In that study, convergent *de novo* evolution of resistance was identified based on haplotype networks at the *ALS* $S_{653}N$ allele, but persistence of standing variation could not be ruled out as responsible for resistance alleles at *ALS* $G_{654}E$, since the allele had been previously reported in weedy populations (Sales et al. 2008). The present study reveals that just over half (56.7%) of regional samples are of crop-weed hybrid origin (22Hybrid BHA, 22Hybrid SH, and 22Triple Hybrid). Thus, while crop-to-weed introgression remains a major driver of weedy rice evolution, other processes, such as *de novo* evolution, the expansion of standing variation, and/or gene flow, apparently have a much larger role than previously inferred. The region-wide persistence of unhybridized weedy rice (30.9%) is also interesting, given that the majority of them (38/49) contain at least one of the known resistance mutations at *ALS*. Unfortunately, the reduced-representation SNPs provided by GBS in the present study are insufficient for the determination of whether these weeds represent additional instances of *de novo* evolution of resistance, as was uncovered in our previous study; to address this question, gene sequencing and haplotype analysis should be pursued with these samples. Finally, it is noteworthy that results from Greene County in the present study (the sole county investigated in Wedger, Roma-Burgos, and Olsen (2022)) indicate 100% ($n=6$) hybrid ancestry with IMI resistance alleles. This finding, which is consistent with the previous findings, suggests that the limited geographical sampling of the earlier study was inadequate to capture much of the genetic diversity present in southern US weedy rice.

In the case of QPE resistance, we are the first, to our knowledge, to document genome-wide evidence consistent with crop-weed hybridization as the origin of the $L_{1781}I$ resistance allele in weedy rice populations. It should be noted that our estimates of QPE resistance are conservative, as we only assayed for the known Provisia HR mutation, which likely differs from that of Max-Ace cultivars (González-Torralva and Norsworthy 2023). While the González-Torralva and Norsworthy (2023) survey of QPE resistance in weedy rice identified $L_{1781}I$ as the primary mutation responsible for QPE resistance, further screening and the inclusion of phenotyping assays for herbicide resistance could help to shed light on the pervasiveness of QPE resistance in contemporary weedy rice populations.

The combination of crop-to-weed introgression and strong selection for the survival and reproduction of hybrids in the face of herbicides has led to a new paradigm of weedy rice research in the southern US that must be considered in further investigations. Expanded sampling in the present study fills in the knowledge gaps left by our previous study. We find that: (1) there is relatively equal representation of SH- and BHA-like samples, in contrast to the BHA-like dominance of 2018 samples; (2) region-wide *Loter* results recapitulate the previously documented bias against crop alleles, but only in BHA-derived populations; (3) both SH and BHA genomically pure weedy rice have persisted alongside crop-weed hybrids, as opposed to the previously observed absence of pure BHA in 2018 samples and (4) contemporary weedy rice shows evidence of diverse genetic mechanisms of persistence in the face of herbicide applications. Beyond these basic research insights, these findings may hold applied value for farmers in field management decisions. In our field collections of weedy rice, which are far from comprehensive, we uncovered fields ranging from 0% to 100% resistant to IMI herbicides. To the extent that individual farmers can be aware of the genotypic composition of their own fields, this could inform and improve management decisions regarding cultivar choice, herbicide deployment and rotation strategies.

Author Contributions

M.J.W. coordinated multi-state collection trips, designed and performed G.B.S. sequencing, designed PCR-based resistance genotyping, analysed data and wrote the manuscript. E.X. helped design and performed and analysed *ACCase* genotyping. T.R.B. scouted and facilitated collection trip in Arkansas. J.L.C. scouted and facilitated collection trip in Missouri. L.C.W. scouted and facilitated collection trip in Louisiana. K.M.O. provided funding and lab/computing resources required for analysis, was involved in the designing and

performing of research and helped in analysing the data and writing the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The new sequence data generated and analysed in this manuscript are available in the GenBank genetic sequence database. BioProject ID PRJNA1172619: Accessions SRX26373856-SRX26374031. Related metadata can be found in the same BioProject. Remaining seeds from collection will be made freely available upon request. Custom python script written for data analysis can be found at https://zenodo.org/records/13844531.

Benefit-Sharing Statement

No international collaborations were required for this research, but benefits from this research accrue from the sharing of our data and results on public databases as described above.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.